STUDIES ON THE PHYLLOPLANE MICROFLORA OF *PINUS RADIATA* D. DON AND ITS INTERACTION WITH THE FUNGAL PATHOGEN *DOTHISTROMA PINI* HULBARY

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PREFACE

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The scheme for partial characterization of yeasts was adopted after consultation with Dr M. I. di Menna and Miss J. M. Wright of the Ruakura Agricultural Research Centre of the Department of Agriculture.

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TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

1. The epiphytic microflora of *Pinus radiata* needles was investigated. Bacteria occurred in the order of 10^6 per gram fresh weight of needles, yeasts 10^4 and moulds 10^3 per gram.

2. Bacterial numbers were higher on shaded aspects and near the bottom of the tree. Numbers also increased as the needles aged although no change was observed as the trees aged. Bacteria were most numerous in late Spring and numbers decreased during the Summer months. However little variation in numbers was noted between the three localities studied. In contrast the population of moulds decreased during Spring increasing to a maximum in Winter.

3. The most common bacteria belonged to the plant coryneform group, followed by lactic acid bacteria and Gram-negative rods (pseudomonads, flavobacteria and paracolons). Low numbers of cocci were also isolated in most studies. Most yeasts on the needle surface belonged to the family Cryptococcaceae. The most regularly occurring sporing moulds were *Cladosporium* and *Penicillium*. Sterile mycelial moulds were also numerous.

An initial study of the phylloplane microflora as an interacting population was made both by removing part of the natural microflora and by introducing micro-organisms from an outside source. The subsequent readjustment of the populations suggested that interactions between micro-organisms on the needle surface played an important role in determining the composition of the

phylloplane population.

4. During the development of needles the epiphytic microflora was initially composed entirely of Aureobasidium pullulans. This was followed by a mixture of Gram-negative bacteria, yeasts and moulds, and finally Gram-positive bacteria became more numerous until the composition of micro-organisms in the phylloplane was that of the mature needles.

5. It is suggested that the most important factor governing the number of micro-organisms in the phylloplane is the availability of nutrients leached on to the needle surface. Under the conditions studied, physical factors such as temperature and humidity had direct effects within the limits set by the availability of nutrients, and indirect effects where they may have influenced the leaching of nutrients from the plant.

6. Preliminary studies were made of the interactions between epiphytic micro-organisms and Dothistroma pini. In culture some saprophytic flavobacteria and pseudomonads inhibited the growth of D. pini. Studies on detached needles suggested that several bacteria from the phylloplane reduced germination of D. pini conidia and that some flavobacteria may retard the subsequent growth of the germ tubes. Later observations suggested that growth of mycelium over the needle surface was not markedly reduced. However field trials on seedlings demonstrated that those sprayed with some saprophytic flavobacteria showed a reduction in symptom expression.

CHAPTER ONE

INTRODUCTION

MICRO-ORGANISMS IN THE PHYLLOPLANE

The presence of an often large and complex saprophytic microflora on growing leaves is now well accepted. The term "phyllosphere" for the leaf surface as an ecological niche was suggested almost simultaneously by Last (1955) and Ruinen (1956). However by analogy with the root surface the term "phylloplane" is more correct since the organisms occur on the leaf surface not in the zone around it (Kerling, 1958).

Micro-organisms isolated

Most investigations have been limited to a single group of micro-organisms but Kerling (1958) counted bacteria, yeasts and filamentous fungi on Beta vulgaris noting changes in these throughout the year. Ruinen (1961) carried out a similar survey on a variety of tropical leaves and Hislop and Cox (1969) noted seasonal changes in bacteria, yeasts and moulds on apple leaves.

These reports suggest that bacteria are the most numerous organisms in the phylloplane (10^5 - 10^{12} per gram fresh weight of leaves). Yeasts also are numerous (10^1 - 10^5 per gram fresh weight of leaves), while moulds occur regularly in variable numbers.

Most epiphytic micro-organisms from leaf surfaces have the following three features in common.

(i) They are able to grow on a wide variety of

host plants (Vosnyakovskaya and Khudyakov, 1960).

(ii) They are widely distributed: similar micro-organisms are found in temperate zones of both the Northern and Southern hemispheres (Last and Deighton, 1965).

(iii) A high proportion of the bacteria are pigmented (Stout, 1960).

Bacteria.

Some of the earliest reports of epipthytic bacteria on leaf surfaces are those by Burri (1903) and Düggeli (1904). They surveyed a wide range of plants in Switzerland and found Bacterium herbicola aureum (Erwinia herbicola, Dye, 1969) to be the most common followed by Pseudomonas fluorescens (Last and Deighton, 1965). Recent work in which more diverse media were used showed that a greater range of bacteria occurred (Gibson et al., 1958; Stout, 1960; Vosnyakovskaya and Khudyakov, 1960). The use of different methods of isolation and systems of identification has however, complicated the interpretation of results from different studies. Despite this the following facts emerge from these studies. Populations of leaf bacteria include a high proportion of pigmented forms, are usually aerobic, and include Gram-positive and Gram-negative types. The Gram-positive isolates include coryneform bacteria, lactic acid bacteria and cocci (Gibson et al., 1958). Gram-negative bacteria include pseudomonads, xanthomonads, flavobacteria and coliforms.

Some regional differences in distribution of bacteria do occur. Spores of the nitrogen fixing bacteria Beijerinckia

are commonly found on foliage in the wet tropics including Indonesia (Ruinen,1956) and Ghana (Meiklejohn, 1962). In contrast Erwinia herbicola has been repeatedly found in temperate zones but not in the tropics. Mack (1936) suggests these differences may be caused by properties of the host since E. herbicola was isolated from temperate zone plants in the Hamburg Botanic Gardens, but not from tropical species in the same locality.

Yeasts.

The yeasts most commonly isolated from leaf surfaces are representatives of the Cryptococcaceae and Sporobolomycetaceae. Di Menna (1959) examined yeasts from pasture plants in various areas of New Zealand and found Rhodotorula and Sporobolomyces to be most common, although Cryptococcus and Torulopsis also occurred regularly. Colonies of Bullera and Hanseniospora were also found. Ruinen (1963) isolated yeasts from tropical leaves. Her isolates were predominantly members of the Cryptococcaceae and included many lipid producing forms. Members of the Sporobolomycetaceae were comparatively rare.

Moulds.

The most widely occurring mould on the surface of green leaves was Cladosporium (Hollomon, 1967; di Menna and Parle, 1970; Dickinson, 1967). Others included Penicillium, Alternaria, Botrytis, Cephalosporium, Stemphylium, as well as a number of genera occurring only under specific conditions.

Factors affecting distribution

Host plant.

Vosnyakovskaya and Khudyakov (1960) noted that most of the numerically important micro-organisms from the phylloplane were not host specific. However some changes in relative importance of different organisms occurred on different hosts. Stout (1960), Kroulik *et al.* (1955) and Ruinen (1961) noted changes in the proportions in which different kinds of bacteria colonized differing hosts. Changes in the numbers of different fungi were noted by di Menna and Parle (1970) on clover and ryegrass. A few host specific forms were also identified.

Host nutrition.

Last (1955) noted that addition of nitrogen, phosphorus and potassium fertilizers to wheat crops increased the incidence of *Sporobolomyces* on the leaf surface.

Season and age of plant.

Most of the studies of epiphytic micro-organisms on leaf surfaces have been on annual plants or deciduous trees where the cycle from leaf formation to senescence takes place in one season. Kerling (1958, 1965) found a progressive increase in the number of micro-organisms on the leaves unfurled until senescence.

Hudson (1962), Hudson and Webster (1958), Hogg and Hudson (1966), Hogg (1966) and Dickinson (1965) studied the micro-fungal succession on ageing leaves of a variety of hosts and found the greatest diversity of fungi on leaves immediately

before and during senescence. Kroulik et al. (1955) found a greater variety of pigmented bacteria on foliage in summer than in winter, and Ruinen (1956) noted an increase in bacterial numbers as leaves aged. A similar trend was noted for bacteria by Stout (1960). The yeast Sporobolomyces did not occur for some time after the leaves unfurled. Last (1955) also noticed this lag and reported that, regardless of the time of year, few yeast colonies were found on cereal leaves until they were past the half way stage of their lives. Aureobasidium pullulans however was found on very young tissues by Hudson and Webster (1958).

Some of these differences may be correlated with seasonal fluctuations of spores in the atmosphere (Last and Deighton, 1965). However there are few reports of the role of the physical environment and host metabolism in determining the size and composition of the phylloplane population.

From this brief survey of the literature it is apparent that information has accumulated about various aspects of the phylloplane population. However most studies have been investigations of one particular aspect, reflecting the interests of the author. This has resulted in a collection of unrelated data. There is need for a co-ordinated investigation of the complete phylloplane microflora of a particular host, its development and the factors affecting its size and composition.

In the present study the first aim was to determine some of the physical factors influencing the saprophytic population on the needles of Pinus radiata D. Don. This work then formed the basis for further investigation of the interactions between micro-organisms in the phylloplane and their possible

role in disease control.

INTERACTIONS IN THE PHYLLOPLANE

Micro-organisms growing on the leaf surface may themselves alter the habitat in several ways, thus playing an important role in determining the composition of the population.

Competition for nutrients

Tukey (1966) has shown that nutrients "leached" on to the surface of plants contain all the mineral and organic materials necessary for life, the amounts varying with the host plant, its condition and stage of growth and the environment. The amount of nutrient present on the leaf surface at any one time therefore varies as the above factors change. Leaching was defined by Tukey and Tukey (1962) as:

> "the loss of organic and inorganic metabolites from above-ground plant parts by the leaching action of aqueous solutions including rain, mist and dew".

They stated that various terms in the literature to describe this phenomenon included exosmosis, lixiviation, guttation, secretion and cuticular excretion. However Tukey (1966) considered leaching a more inclusive and more useful word as it places emphasis on the process itself rather than upon the mechanisms and products involved.

If nutrient supply is an important factor determining the number of micro-organisms on the needle surface of Pinus radiata competition for food may be a limiting factor in the

environment. If this is so some balance must be achieved between the nutrient supply and the micro-organisms able to utilize it. Successful exploitation of most nutrients will depend on the ability of the organisms to produce the enzymes to break it down, and to assimilate it. Micro-organisms able to do this rapidly will be at an initial advantage. Winogradsky (1949) has called those micro-organisms able to multiply rapidly on the addition of soluble organic nutrients zymogenous organisms. These he compared with the autochthonous micro-organisms which were able to survive and grow slowly in the absence of readily available nutrients - probably by using more complex nutrients. Micro-organisms in both categories could contribute to the population on the leaf surface.

In any ecological system various nutrients may become limiting. Studies on soil populations suggest that:

(i) Nitrogen may become limiting if the carbon to nitrogen ratio becomes too high (Maurer and Baker, 1965)

(ii) Microbial immobilization of carbon can prevent germination and growth of fungal spores (Maurer and Baker, ibid; Cook and Schroth, 1965).

(iii) In some cases vitamins may become limiting. Erwin and Katznelson (1961) showed decreased growth of Phytopthora cryptogea in the presence of an Arthrobacter isolate which utilized thiamine.

These factors may also be important in the phylloplane.

Modification of host exudates

The microbial breakdown of nutrients into a form suitable for assimilation takes place extracellularly. Thus the initial nutrient supply may be modified by the activity of the micro-organisms utilizing it, the breakdown products from one group of substances providing food for other organisms. This type of succession occurs throughout nature and may be typified by the breakdown of litter by micro-organisms (Saito, 1966; Caldwell, 1963; Kendrick and Burges, 1962).

Many micro-organisms may also produce growth factors and vitamins essential for the existence of others (Lochhead, 1957). Synergism between micro-organisms in the phylloplane can also occur where self-inhibitory substances produced by one organism are removed by the activity of others. Brathwaite and Dickey (1970) showed that a Corynebacterium sp. produced acids (from sugars) which apparently restricted its growth. When Pseudomonas caryophylli was present acid accumulation did not occur and growth of Corynebacterium was enhanced.

It has also been shown (Tokin, 1960; Kuc et al. 1956) that among the substances present on the leaf surfaces are some anti-microbial agents. These may act directly to limit organisms on the leaves. However it is possible that these substances may be altered by microbial activity, either by direct breakdown or by the production of metabolites that render the toxic substances harmless. In this case the presence of some organisms would be dependent on the breakdown of these substances by other micro-organisms.

Production of inhibitory substances

The breakdown products or the metabolic wastes from some micro-organisms may be inhibitory to some other organisms, thus preventing their growth.

Antibiotic production has often been implicated as a factor responsible for microbial inhibition. However in many cases *in vitro* studies have been incorrectly extrapolated to explain observations *in vivo*. The problems in elucidating the importance of this factor have been reviewed by Brian (1957, 1960). The lack of correlation between *in vitro* and *in vivo* studies is mainly because the effects of other micro-organisms on the antibiotic producer or its products are not recognised. For example in soil the production of griseofulvin by *Pencillium nigricans* is reduced by the action of other soil micro-organisms, both by other antibiotic producers and non-antibiotic-producing forms such as *Mucor ramannianus* (Brian, 1957). Studies in the soil have shown that the production of antibiotics in a given habitat may be limited by any of the following factors (Baker, 1968).

(i) Lack of adequate nutrition, especially carbon sources, for the growth of the antibiotic producing organism.

(ii) Breakdown or inactivation of the antibiotic as it is formed.

(iii) Inhibition of growth of the antibiotic producer by other micro-organisms.

Direct attack by other micro-organisms

Waksman (1947) and Mitchell and Alexander (1963) gave another type of reaction by which one organism exerts a

deleterious effect on another. Their study on the lysis of soil fungi by bacteria suggested the existence in the soil of micro-organisms capable of digesting fungus mycelium. This type of interaction may be of importance in the phylloplane. Lloyd and Lockwood (1966) and Garrett (1965) however suggested that the destruction of mycelium may more often be due to autolysis following starvation than to direct attack by other micro-organisms.

Effects on the host plant

Not only do plant substances become available on the leaves but the plants may also reabsorb materials from the leaf surface. Among these may be products of microbial metabolism. For example Libbert et al. (1966) suggested that indole acetic acid (I.A.A.) produced by epiphytic micro-organisms may be absorbed by plants. Lochhead (1957) and Lochhead and Cook (1961) also discussed the capability of the bacterial flora to synthesize growth factors.

Antibiotics produced in the phylloplane may be absorbed by the host plant and in some cases translocated, making the leaf surface unsuitable for the growth of some micro-organisms. However antibiotics also affect the host metabolism. Brian (1957) discussed the effects of a number of antibiotics on plants. Most have been found to be phytotoxic to some extent.

Since products of microbial growth may include substances both beneficial and harmful to plants they may, by either retarding or promoting growth of the host, influence the nutrients produced and leached from the leaves, and in turn bring about changes in the microbial population.

Another reaction of the host plant to some saprophytic micro-organisms is the formation of anti-microbial substances. The production of these 'phytoalexins' has been described by Muller (1958), Gaumann, (1951, 1954), Kuc et al. (1956) and Cruickshank and Perrin (1961, 1963).

A direct effect on the host was described by Ruinen (1966) who showed the host cuticle to be decomposed by micro-organisms, thus providing food for further organisms and opening up new habitats.

From this survey of possible interactions in the phylloplane it is apparent that if nutrients are limiting, interactions between the micro-organisms and with the host plant will result in the development of a microbial population in dynamic equilibrium with the environment, the host plant and itself. If this is the case some reaction would be expected by the population to the introduction of new organisms. Such introductions are continually happening as a result of deposition from the air, washings from other needles and replication of established organisms.

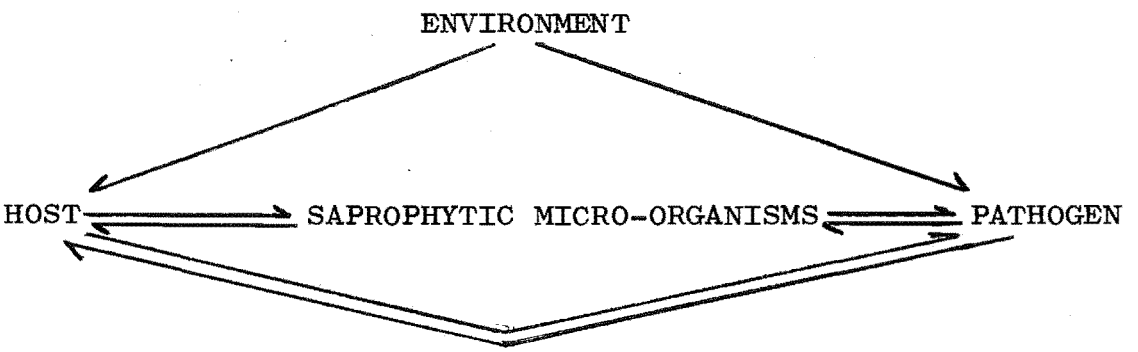
The experimental work described here was designed to show whether the population on the needle surface was simply a function of the host and the environment, or if there was sufficient competition for the build up of an interacting and interdependent population of micro-organisms.

INTERACTIONS BETWEEN SAPROPHYTES AND PATHOGENS

The presence of a population of micro-organisms on the leaf surface is important in the development of leaf diseases since many potential leaf pathogens must grow on the leaf

surface prior to infection. It must therefore take its place in the population already existing there interacting with, and being influenced by, the other micro-organisms already established.

Thus the development of disease in a plant is not simply the result of interaction between the pathogen, the host and the physical environment, but a fourth factor, the saprophytic microflora must be considered. These relationships may be illustrated as follows:



The ways in which micro-organisms on the leaf surface may modify the habitat and so change the microbial population have been discussed. These interactions involve all organisms on the leaf surface whether they be saprophytes or pathogens. Theoretically almost any part of the life cycle of a pathogen, or of its interactions with the host could be inhibited by microbial saprophytes.

Saprophytes may directly antagonise a potential pathogen but more commonly reduce the inoculum potential, i.e. inhibit the local build up of energy required to initiate and maintain an invasion of host tissues. On the leaf this could result from:

(a) inhibition of fungal spore germination

(b) inhibition of growth of mycelium of fungi

(c) inhibition of the accumulation of sufficient numbers of bacteria to effect entry of the host tissues.

Following infection by a pathogen, development of the disease and the production of propagules could be inhibited by epiphytes simultaneously occupying the portion of the leaf inhabited by the pathogen.

To these possible direct actions may be added indirect actions of saprophytes on pathogens. A saprophyte may either reduce or induce a plant resistance response that also repulses the pathogen, or it may otherwise alter the physiology of the plant so as to influence infection or the course of the disease.

INTERACTIONS BETWEEN SAPROPHYTES AND PATHOGENS

Control of disease by saprophytic micro-organisms

Most of the early work on the use of microbial antagonists to control leaf diseases consisted of *in vitro* studies where known antagonists were tested against pathogens. This work was reviewed by Wood and Tveit (1955).

One of the earliest suggestions that the epiphytic microflora might play an important part in disease resistance came from Simmonds (1947). He used bacteria isolated from wheat culms in studies of the wheat seedling disease caused by *Helminthosporium sativum* and concluded that part of the bacterial flora on the plant and seed was inhibitory to this pathogen. Since then many workers have reported successful control of disease of various plants using epiphytic micro-organisms.

Pathogens entering through wounds

Bier and Rowat (1962) found that Epicoccum nigrum isolated from nodes and lenticels of Populus trichocarpa inhibited Hypoxylon pruinatum in dual culture. They also showed that organisms from unsterilized barkwater suspensions prevented canker formation on greenwood cuttings, providing the bark turgor of the cuttings was high.

A similar observation was made by Kristic and Hocevar (1959) who found that bacterial antagonists applied to wounds reduced the incidence of chestnut blight caused by Endothia parasitica. Teliz-Ortis and Burkholder (1960) reported the reduction of bean blight by a Pseudomonas fluorescens isolate derived from tissues of diseased plants. In dual culture this organism inhibited a number of bacterial pathogens, and application of this isolate prior to wound inoculation with Pseudomonas phaseolicola greatly reduced disease. They believed the inhibition was the result of antibiotic production and that the antibiotic could be translocated in the plant. Farabee and Lockwood (1958) isolated yellow pigmented saprophytic bacteria from apple fire blight cankers and showed inhibition of the disease when these were inoculated into wounds simultaneously with Erwinia amylovora. Other reports of non-pathogenic yellow bacteria reducing canker formation on various plants have been published (Billing and Baker, 1963; Goodman, 1964; Riggle and Klos, 1970). In this connection Crosse (1959) mentioned a common epiphytic bacterium isolated from healthy cherry leaves which reduced disease incited by Pseudomonas mors-prunorum.

Pathogens entering intact tissues

Pathogens entering intact tissues or natural openings are

widespread. In the U.S.S.R. Khudyakov and Kozlov (1958) isolated a few epiphytic bacteria from plant surfaces. These became established on grapevines and reduced the incidence of powdery mildew. Leben (1965) reported that a bacterial isolate from the leaves of cucumber seedlings was antagonistic to fungi in dual culture, and reduced cucumber anthracnose on seedlings, Alternaria solani on tomato seedlings and Trichometasphaeria turcica on maize seedlings. It had however no effect on powdery mildew. An antibiotic was produced by this isolate but its importance in antagonism was not substantiated. Another example is provided by McBride (1969) who demonstrated a reduction of Douglas Fir needle rust caused by Melampsora medusae when sprayed with Bacillus spp. commonly saprophytic on healthy Pseudotsuga foliage.

Pathogens normally infecting moribund or dead tissues

Some organisms rarely produce disease in vigorously growing plant parts but may multiply in moribund or dead tissues and then invade healthy tissues (Garrett, 1960). Newhook (1957) showed that infection of tomatoes by Botrytis cinerea was reduced when moribund and dead petals were sprayed with spores of some epiphytic fungi commonly found on dead petals. Cladosporium herbarum and a Penicillium sp. were particularly effective. Cladosporium herbarum and Pullularia pullulans also slightly reduced fruit rot in strawberries (Bhatt and Vaughan, 1962) although inhibition depended on the stage of growth at which the antagonists were added. Field trials showed that spraying with spore suspensions of C. herbarum did not decrease rot but did increase the production of marketable berries. Simard et al. (1957) reported antagonism

between the apple scab pathogen Venturia inaequalis and three Penicillium isolates found on dead leaves of apples.

These examples of antagonisms against a variety of pathogens suggest that micro-organisms resident on the leaf surface may play an important role in the control of plant diseases. In particular it seems possible that micro-organisms normally epiphytic on needles of Pinus radiata might reduce infection of those needles by Dothistroma pini.

An investigation of this possibility requires that information be available firstly on the micro-organisms resident on the needle surface and the factors influencing them, and secondly on the biology and ecology of D. pini in New Zealand. The former has been the aim of the first part of this thesis and information on the latter has been published chiefly by workers at the New Zealand Forest Research Institute, Rotorua.

NEEDLE BLIGHT OF PINUS RADIATA CAUSED BY DOTHISTROMA PINI

The pathogen

Dothistroma pini Hulbary (Hulbary, 1941) causes needle blight in many pine species. It was first noted in New Zealand in 1962 (Gilmour, 1965). The perfect stage Scirrhia pini has been described by Funk and Parker (1966) and in New Zealand by Bassett (1967).

Gibson et al. (1964) stated that D. pini is a slow growing fungus with simple nutrient requirements. They also noted that it was not a successful competitor with other saprophytic organisms and that it therefore owes its continued existence to an ability to invade pine foliage. Sanders (1969) studied

the optimum conditions for growth in culture. He showed that germination occurred over a temperature range of 10°C - 28°C with the optimum between 15°C - 27°C. Growth and sporulation occurred over a wide pH range (2.2 - 7.8) with optimum conditions pH 4.2 - 5.0. Addition of a variety of nutrients showed high concentrations of sugars to favour growth.

Infection cycle

Dispersal

The fungus is distributed by conidia formed in large numbers in stromata in infected foliage. The spores are liberated when stromata come into contact with liquid water, which provides the means for their dispersal. During rain, drops laden with spores may drip on to lower foliage or to the ground, where the conidia may be dispersed in splash droplets. Evaporation of these take-off droplets may cause the spores to become airborne. There is some evidence that, under special circumstances, clouds may provide a means of long range dispersal (Gibson *et al.*, 1964). However Jancarik (1969) noted that in many cases the first introduction of infection into an area is from infected needles either blown into the area, or introduced mechanically (for example on people, vehicles and equipment).

Secondary spread occurs mainly through rain and dew carrying conidia to foliage. Jancarik (*ibid*) has shown that in a nursery the disease spread at least 175-200cm from an initial infection locus in one growing season.

Infection

Infection of young trees can occur at any stage of growth. However in P. radiata there is a marked decrease in susceptibility to needle blight with increasing age (Jancarik, 1969; Gibson, 1965). Gibson reports that stands over fourteen years of age (and at times younger than this) are markedly resistant to initial infection, and to the internal spread of the fungus after infection, and suggests that this is linked with an intrinsic change in the foliage of older trees.

Gadgil (1967) studied the infection process and reported that once conidia land on the needle surface, germination usually takes place after one to three days. The resulting mycelium may produce secondary conidia which provide an additional source of inoculum. Mycelium was seen to spread over the needle surface and after ten days produced appressoria covering the stomatal openings and filling the stomatal cavities. From these appressoria a narrow infection peg penetrated between the guard cells and branched out into the stomatal chamber. Direct penetration of the epidermis was observed only when macerated mycelium rather than spores was used as the inoculum.

Following infection the mycelium permeated the surrounding mesophyll tissues, also penetrating the resin canals. Hyphae were both intra- and inter-cellular. After about sixteen days the infected mesophyll began to disintegrate.

The time taken for disease symptoms to appear after infection has taken place varies in both nurseries and forest stands (Jancarik, 1969). In the summer it is about two months, but during the winter no symptoms may be seen for 4-5 months or more.

Gadgil (1967) showed that on inoculated seedlings stromata were produced $3\frac{1}{2}$ - $4\frac{1}{2}$ months after inoculation. The stroma primordia arose in the mesophyll and conidia were borne over the whole exposed surface of the ectostroma on simple densely packed conidiophores. There was considerable disorganization of the mesophyll in these areas.

The first visible symptoms were seen at about this time. These were small necrotic patches on the needles. A few days after the appearance of these lesions shining black stromata burst through the epidermis. One or many fruiting bodies were present in a single lesion. The lesion gradually turned reddish brown and the red band typical of the disease was produced.

Gadgil (ibid) observed that in transverse sections of diseased needles mesophyll cells were disorganized and killed in advance of hyphal growth. This suggested the possibility of exotoxin or exo-enzyme production by the hyphae. A toxin has since been isolated by Bassett and purified and characterized by Batt (Bassett, 1970).

Effect of environment

Rainfall

The studies of Murray and Batko (1962) have shown that in Dorset the intensity of needle blight attack can vary considerably with early summer rainfall. This is also true in East Africa where severe needle blight was found to be associated with an evenly distributed rainfall of more than 137 cm per annum, with a decline in severity as the rainfall dropped to 75 cm per annum (Gibson, 1965). In New Zealand,

Gadgil (1968) investigated the effect on infection by Dothistroma of leaf wetness periods of 8, 16 and 24 h. The result from these experiments were erratic except that at 80°C infection increased from 43% after 8 h wetness to 80% after 24 h.

The moisture factor appears to be a complex one involving a minimum moisture requirement over a variable period. Gibson et al. (1964) suggested that even in areas of low rainfall, long periods of drizzle may result in even more severe blight damage than shorter periods of high rainfall.

While water is important for spore germination and penetration of the needles, dry conditions favour the survival of the pathogen on dead needles - possibly because these conditions do not favour secondary invasion by saprophytes.

Temperature

Gadgil (1967) stated that in trials at Rotorua infection increased with increasing temperature from 10°C to 27°C. Gibson (1965) suggested a narrower range of temperatures for germination of conidia in East Africa (12°C - 22°C) and noted that moisture must be present. He also noted that fluctuations outside this range may delay but not inhibit germination.

Effect of shade

Gibson et al.(1967) mentioned that both in the field and in the laboratory susceptibility of P. radiata foliage to attack by D. pini was increased in sunlight, and that infected needles also sporulated more profusely in sunlight.

The infection of P. radiata needles by Dothistroma pini

is controlled by many factors some of which appear to be environmental. However it is possible that such physical factors act indirectly through their effect on micro-organisms saprophytic on the needle surface. These may in turn either stimulate or inhibit the germination and growth of D. pini in this habitat in any of the ways described previously.

The following aspects were considered in this investigation of the phylloplane microflora of P. radiata and its possible interaction with the fungal pathogen D. pini.

1. Numbers of micro-organisms present and their occurrence under different environmental conditions.
2. Characterization of the isolates from the above study.
3. The distribution of micro-organisms on the needle.
4. The phylloplane microflora as a population.
5. Colonization of the emerging needles.
6. Factors influencing the phylloplane population.
7. Interactions between these micro-organisms and D. pini.

CHAPTER TWO

GENERAL METHODS AND MATERIALS

ENUMERATION AND ISOLATION OF BACTERIA YEASTS AND MOULDS

Collection of specimens

Needles from Pinus radiata trees were collected from Bottle Lake plantation in Burwood on the outskirts of Christchurch. Trees on the edge of the plantation or bordering access roads were avoided, as these were presumably subject to climatic variations not encountered within a large stand of trees. Needles sampled to develop techniques were collected from trees growing near the University.

Since the purpose of the survey was to determine the composition of the microbial population on the surface of healthy needles, only green needles without discolouration or injury were collected. The harvested needles were placed in plastic bags and taken to the laboratory. Experimental work was begun within an hour of collection.

Isolation of micro-organisms from the needle surface

Several methods of isolating micro-organisms have been used by various workers. All have limitations and the technique selected is usually the one that gives the greatest amount of information on the particular aspect under study. This perhaps is unfortunate as in many cases the use of different techniques prevents the comparison of results obtained from studies in allied fields. The whole field of interactions between micro-organisms on plant surfaces appears,

however, to be so complex that some specialization in approach and methods must occur if we are to learn anything of value.

Dilution plate technique

Methods of sampling organisms from the soil have been reviewed by Durbin (1961) and Menzies (1963). Many of the considerations applicable to the soil also apply to other complex habitats such as the needle surface. In spite of valid objections to the use of agar dilution plates as a means of enumerating and characterizing the microflora, this is a simple technique and no other single method has been devised which offers significantly better results while giving as much information about the organisms present.

Isolation from needles. The needles were given a 3 - 5 s initial wash in sterile tap water. A test showed that during this time 200 bacteria per gram were removed whereas 2,000 sporing fungi were dislodged. These results are shown in Table I.

Table I. Numbers of micro-organisms removed per gram of needle in initial wash.

Number of colonies x 10^{-2} per gram fresh wt.					
Cladosporium	Penicillium	Other fungal spp.	Non-Sporing fungi	Yeasts	Bacteria
12	6	2	0	1	2

This initial wash was considered desirable as it allowed the recovery from plates of the less prolific moulds that were otherwise over-run by the heavily sporing species. Other tests demonstrated that maceration of the needles and subsequent dilution was a more effective means of isolating both bacteria and fungi than washing needles. In these tests washing needles was carried out as follows: Two grams of needles were given an initial 3 - 5 s wash and placed in screw capped 500 ml bottles with 100 ml of sterile tap water and 10 g of glass balls and shaken vertically by hand for 2 min and then placed on a wrist action shaker for a further 10 min. The needles were then transferred to another similar bottle and shaken as above. This process was repeated through ten changes of sterile water. From each bottle a dilution series was prepared and five replicates of 10^{-1}, 10^{-2}, and 10^{-3} plated on each of Martin's Rose Bengal, streptomycin medium (Martin, 1950) and Nutrient agar (Difco) for enumeration of moulds, and bacteria respectively. Figure 1, p 18 shows the effectiveness of washing needles in removing micro-organisms from the needle surface. It is apparent that, even after washing in nine changes of sterile water, micro-organisms remained attached to the needle surface. This was checked by plating the washed needles on Martin's medium and nutrient agar and observing the subsequent growth of micro-organisms. In all cases colonies developed in the agar along the needles.

Maceration should ensure that all the micro-organisms on the needle are included in the initial suspension.

The validity of the maceration technique depends, however upon the absence of micro-organisms inside the healthy needle.

This was tested by dissecting off the epidermis under sterile conditions and plating the internal tissues on the media described for bacteria, yeasts and moulds. No micro-organisms grew on any plates so it was assumed that the internal tissues of healthy needles were free of micro-organisms.

Chan and McManus (1967) however have suggested that maceration of tissues may kill a significant proportion of the resident bacteria. It is difficult to compare microbial counts from washed and macerated needles since in the former method the micro-organisms are removed in successive washes whereas in the latter all micro-organisms are found in the resultant suspension. However Fig. 1 suggests that serial washing of needles dislodged 75% of the removable bacteria in the first wash and 85% after two washes.

On this basis an experiment was set up to compare the effectiveness of serial washing and maceration of needles in enumerating bacteria on the needle surface. Five 4 g samples of needles were given a brief initial wash in sterile water. From each sample 2 g of needles were washed in three changes of sterile tap water diluted and plated in nutrient agar as described above. After 14 days incubation at 25°C the bacteria appearing on the plates were counted and the total number removed during the three washes calculated.

The remaining 2 g of needles from each sample were then transferred to 100 ml of sterile tap water and comminuted in a "Virtis 45" Homogenizer for 10 min at medium speed (approximately 12,000 rev/min) The resulting suspension was then serially diluted in sterile tap water and five replicates of 10^{-1}, 10^{-3}, and 10^{-4} plated for each sample. Table II gives the number of bacteria removed by each method.

Table II. Numbers of bacteria removed by washing and macerating needles.

Number per gram fresh weight of needle x 10^{-5}		
Sample	Washing	Maceration
A	7.0	87
B	8.9	98
C	9.8	147
D	7.2	74
E	8.3	92

From this table it is apparent that a greater number of bacteria are removed from the needle surface by maceration. If we assume that the figures given represent between 80% and 90% of those propagules able to be washed off needles it is apparent that mortality of bacteria caused by maceration is less important than the inefficiency of the washing technique.

The isolation technique used throughout this study therefore was as follows. Three replicates of each sample to be examined were studied separately. In each replicate 2 g of needles were given a brief (3-5 s) wash in 100 ml sterile tap water, transferred to a further 100 ml and macerated. The resulting suspensions were then diluted as described above and plated as described later in this chapter.

Diluents. Straka and Stokes (1957) reported rapid and extensive destruction of bacteria in distilled water and tap water and recommended the use of 1% peptone water as a diluent

to prevent this mortality. Stout (1960) however compared the recovery of bacteria from herbage samples in New Zealand using both sterile tap water and peptone water as diluents. His results showed that under the conditions he employed there was no significant advantage in the addition of peptone. In this study, where techniques were very similar to those used by Stout, sterile tap water was used to dilute the samples.

Media. It is recognised that no single medium will provide all the conditions required for growth of all micro-organisms from a complex habitat (Jefferies et al., 1953; Lockwood, 1959; Robinson, 1962).

Artificial media selected to isolate and count the micro-flora must provide suitable conditions for the growth of as wide a variety of micro-organisms as possible. For this reason several media were tested so that the groups selected against were kept to a minimum. Bacteria, yeasts and moulds were tested separately as each group has its own range of growth requirements.

Bacteria: The media tested for support of bacterial growth were:

(i) Soil extract agar (Bunt and Rovira, 1955) shown by Robinson (1962) to be the least selective medium of those tested for soil bacteria

(ii) Soil extract agar with the addition of 10 ml per l needle leachate

(iii) Nutrient agar (Difco)

(iv) Nutrient agar with the addition of 10 ml per l of needle leachate.

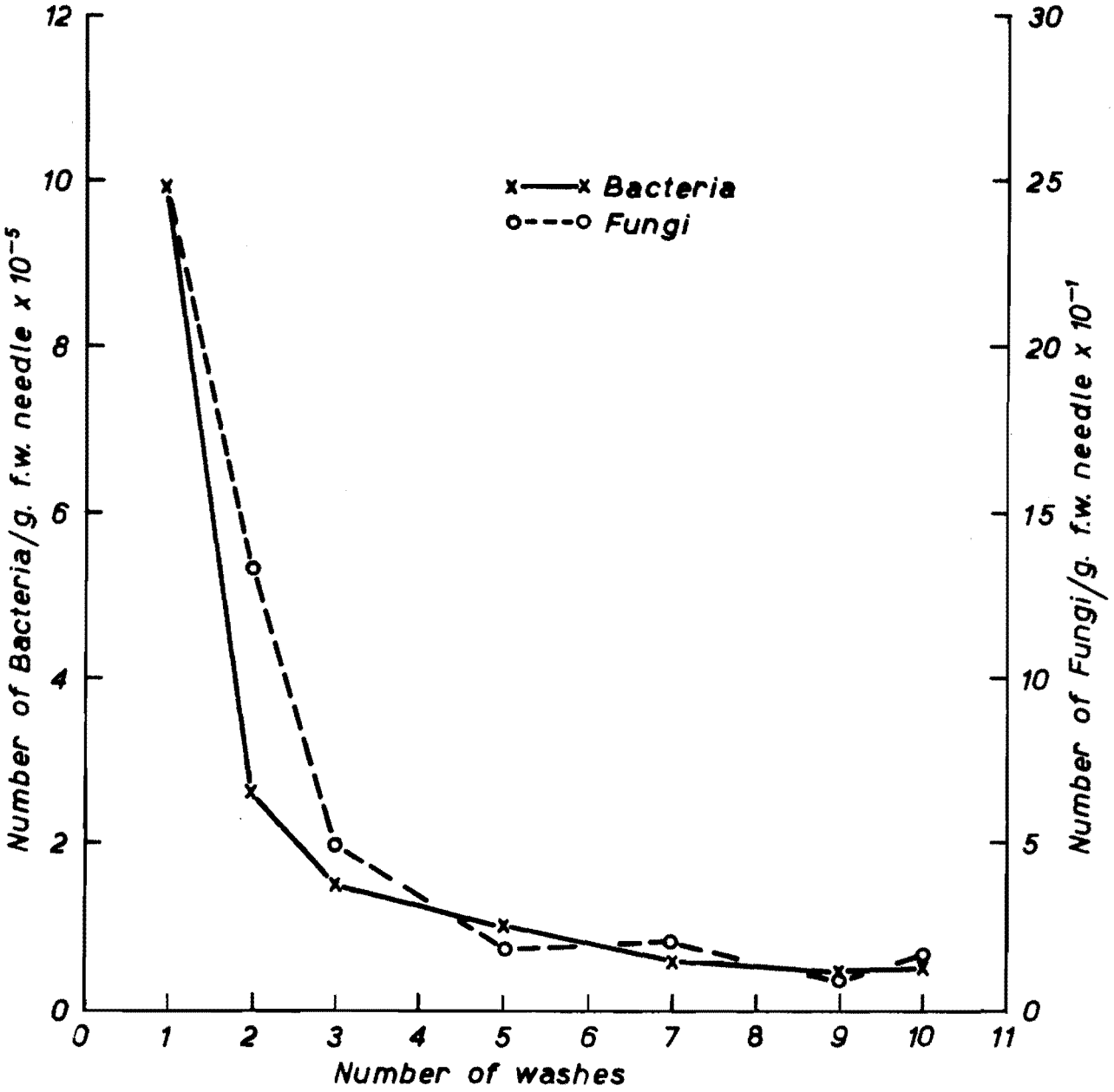


Fig.1—Number of Micro-organisms removed from needles by Serial Washing

The needle leachate was prepared by soaking 10 g of needles in 100 ml of sterile water for 24 h followed by filter sterilization of the liquid.

Data from these tests are given in Table III and show the media to which needle leachate had been added to allow the isolation of the greater number and variety of bacteria.

For the isolation of bacteria the soil extract agar/ needle leachate medium was selected.

Table III. Numbers of bacteria isolated on different media

Medium	Numbers of bacteria $x10^{-5}$ per g of needle						
	Total	Coryne-form	Lactic Acid	Pseud-omonads	Flavo-bacteria	Para-colons	Cocci
Soil Extract Agar	37	29	3	1	1	2	1
Soil Extract Agar and Needle Leachate	98	86	3	2	2	3	2
Nutrient Agar	32	27	1	0	2	2	0
Nutrient Agar and Needle Leachate	91	82	2	1	3	2	1

Yeasts. The medium selected for growth of yeasts was a glucose, peptone agar found by di Menna (1959) to be most satisfactory for the isolation of yeasts from the leaves of pasture plants in New Zealand.

Moulds. The media tested were:

(i) Malt extract agar. (Difco malt extract or "Maltexo" 2%, agar 1.5%).

(ii) Rose Bengal, streptomycin medium (Martin, 1950) reported by Kotheimer and Christensen (1961) to allow recovery of a greater number of fungal genera from barley kernels than any other medium.

(iii) The medium of Jefferies *et al.* (1953) which is suitable for the growth of organisms requiring ammonium or amino-nitrogen.

The results of these tests which are given in Table IV showed that Martin's Rose Bengal medium allowed the recovery of greater numbers and more kinds of fungi than either of the others.

Table IV. Numbers of moulds isolated on different media

Medium	Total number x 10^{-3} per gram needle	Number of genera
Malt Extract Agar	49	6
Martin's medium	79	10
Jefferies *et al.* medium	54	5

Enumeration and culture

The use of the above method where the microbial population is determined relative to a known fresh weight of needle is open to criticism (Allen, 1970) on the basis that changing moisture content of plant material growing under different conditions may alter the relative microbial counts. To test this factor needles were collected from trees under various conditions, weighed, dried at 100°C for 12 h and re-weighed. The results were as follows:

Table V. Moisture content of needles grown under different conditions

Source of needles	% Moisture
Nursery grown seedlings maintained at 26.7°C, 100% R.H.	66.0
" " 10.0°C, 95% R.H.	66.1
" " - 1.0°C, 30% R.H.	66.0
Mature trees, 1 month old needles	65.9
" " 14 month old needles (shaded)	62.5
" " 14 month old needles (sunny)	62.7

These results justify the use of fresh weights as a means of comparing microbial colonization of needles under varying conditions, since the only factor affecting moisture content appeared to be the age of the needle. This is understandable as the needle has a rigid structure and cannot swell very much.

Therefore there is little moisture increase in times of good water supply. However if the moisture level falls below the maintenance level the needle dies. This is prevented partly by the small surface : volume ratio, the corrugated surface and the sunken stomata of the needles.

Bacteria. By isolating and identifying micro-organisms from the leaf surface we are endeavouring to represent as nearly as possible the composition of the original population. The greater the number of organisms isolated from each needle the greater the chance there is of all kinds being represented. All isolates must be characterized and many tests are required to classify bacteria but both time and facilities are limited. So some compromise must be reached between the demand for as large a representation of the original population as possible and the need to be able to characterize the organisms so they might be recognized in future work. Therefore from each sample dilutions producing between 30 and 100 colonies per plate were counted and recorded. From the same plates 50 colonies were then taken and transferred aseptically to plates of nutrient agar. Where there were less than 50 colonies per plate, two plates were used. Where the number per plate was greater a sector was drawn to include 50 colonies and these were all taken. The colonies were streaked on plates of nutrient agar and single colonies transferred to nutrient agar slants in McCartney bottles (1oz Universal Containers) for storage.

Yeasts. These were counted and isolated in a similar manner to the bacteria except that thirty colonies from each sample were cultured.

Moulds. A suitable dilution was selected and the different kinds of moulds counted and described. One representative of each kind was then transferred to a slant of Langeron's weak potato carrot agar (Plant Pathologists' Pocketbook, 1968) in a McCartney Bottle.

Analysis of plate counts

Cowell and Morisetti (1969) stated that under ideal conditions, because of sampling error in the number of organisms selected in replicate aliquots, the colony counts on replicate plates of the same sample were distributed as a Poisson series.

The conditions that needed to be fulfilled were:

1) That viable organisms were distributed at random throughout the agar
2) That accurate aliquots were taken
3) That each viable organism produced a colony
4) That colonies were counted without error

The fulfilment of these conditions depended mainly on perfection of technique. In addition it was said to be essential that the development of one organism be independent of any other and this depended on the nature of the organisms concerned.

Conflicting reports have appeared in the literature for pour-plate counts. In a review of a number of workers' results Eisenhart and Wilson (1943) concluded that generally a Poisson series was obtained when pure cultures were involved. However mixed cultures on non-selective media did not always conform.

They stated that departure from the Poisson series meant that conclusions drawn from such data were suspect because the mean was unreliable. Fisher *et al*. (1922) had previously developed a chi square criterion (or index of dispersion) to test whether such data did conform to a Poisson series

$$\chi^2 = \frac{1}{\bar{x}} \sum (x - \bar{x})^2$$

where x = the colony count on one plate

$\bar{x}$ = the mean of colony counts on replicate plates

In the present work, this formula was used to test several plate count results to see if they conformed to a Poisson series. The plate counts and chi square variation for bacterial plate counts are given in Appendix I, Table XIX. Loveday (1961) states that values of p above 95% suggest some defect in the medium leading to subnormal variation. Abnormally high variation ($p < 5\%$) was believed to be due to colonies exerting influence on each other. For values of p between 95% and 5% the Null hypothesis holds and the plate counts can be said to represent a Poisson series. This is true for all the counts shown in Table XIX Appendix I.

In a Poisson series the data will never be less variable than that which is due to random sampling alone. An important characteristic of a Poisson series is that the standard deviation is equal to the square root of the mean of the distribution and therefore the count is itself a measure of the precision of the test (Cowell and Morisetti, 1969).

Since the values based on plate counts given in this thesis are direct transformations from the mean of the plate counts (no other variation is introduced) additional statistics

are unnecessary.

Other methods

Membrane filtration

This method was used as a comparison with the maceration - dilution plate technique.

One g of needles was macerated in 100 ml of water as described for the dilution series. The suspension was then passed through a membrane filter, pore size 0.45μm. The filter was then stained with Loeffler's methylene blue (Harrigan and McCance, 1966) and examined microscopically by oil immersion. The bacteria on the filter were counted and the number per g fresh weight of needles calculated. This method showed bacterial numbers in the order of 10^6 to 10^8 per g fresh weight of needles. This is similar to the results obtained from the dilution plates.

TESTS USED TO CHARACTERIZE MICRO-ORGANISMS

Identification of bacteria

Morphological and biochemical tests were used to differentiate the kinds of bacteria found. Not all the tests described below were used on all cultures as some apply only to certain groups of bacteria. Except where stated to the contrary all cultures were incubated at 25°C.

Morphological characters

Cell morphology and motility. Wet mounts were made using 24 or 48 h cultures (depending on the stage of growth) from nutrient broth. These were examined by phase contrast.

Flagellation. 24 h cultures were examined using a Hitachi HS-7 electron microscope. The bacteria were negatively stained with potassium phosphotungstate (Horne, 1965). The stained cells were then mounted on a carbon coated nitro-cellulose grid and examined at a magnification of x9,000 - 12,000.

Acid fastness. Smears from 48 h cultures were stained using the Ziehl-Neelson stain for acid-fast bacteria (Harrigan and McCance, 1966).

Gram reaction. The stain reactions (Skerman, 1967) were recorded as Gram-negative, Gram-positive or Gram-variable.

Cultural characters

Pigmentation. This was observed on the glucose, yeast extract medium (G.Y.C.A.) of Dye (1962).

Production of fluorescent pigments. Production of diffusible fluorescent pigments was observed on medium B of King et al. (1954), but using tryptone (Difco) instead of proteose peptone. Plates were streaked and recorded after 2 - 4 days.

Tolerance to sodium chloride. This was determined in tubes of nutrient broth containing 0, 6 and 10% NaCl. The tubes were examined for turbidity over a period of 10 days.

Utilization of carbon compounds

Mode of utilization of glucose. Cultures were tested using Park and Holding's (1966) modification of the methods used by Hugh and Liefson (1953). These tests were examined twice daily for two days to record oxidative or fermentative utilization of glucose. A final reading was made after four days.

Production of acid from carbohydrates. Phenol red broth (Difco) with the addition of either 1.0% sucrose or 1.0% lactose was used in the initial tests. However this was found to inhibit the growth of some organisms. Also the pK value of phenol red is 7.8 and the production of small amounts of acid could not be detected. For final tests an inorganic medium (Dowson, 1957) with bromothymol blue (pK 7.1) as an indicator was used as the basal medium.

Utilization of cellulose. Isolates were tested for ability to degrade cellulose by adding a strip of filter paper to the inorganic salts medium of Harrigan and McCance (ibid). These were examined daily for three weeks.

Biochemical tests

Hydrolysis of gelatin. The bacteria were stabbed into a tube of nutrient gelatin (Difco) and incubated at room temperature. Growth and liquefaction were recorded periodically for three weeks.

Reduction of nitrate. Nitrate peptone water (Harrigan and McCance, ibid) was used for this test. Duplicate tubes were prepared and after 2 and 5 days tested by adding sulphanilic acid and dimethyl-a-naphthylamine. Zinc dust was added to those cultures with a negative

reaction to demonstrate reduction beyond the nitrite stage.

Action on litmus milk. Bacteria were inoculated into litmus milk (Difco) and incubated for 14 days. During this time the cultures were examined for peptonization, acid production, or alkali production.

Production of catalase. A loopful of bacterial growth from nutrient broth cultures was emulsified in 1 ml of hydrogen peroxide on a clean glass slide. The presence of catalase was denoted by the production of gas bubbles.

Production of oxidase. Kovac's test (1956) as modified by Steel (1961) was used to determine oxidase production.

Identification of yeasts

Production of starch. Cultures were streaked on plates of starch production agar (Lodder and Kreger-van Rij, 1952). After one to two weeks a few drops of Lugol's iodine were dropped on the cultures. The presence of a blue colouration denoted the presence of starch.

Fermentation of glucose. The method of Beech et al. (1968) was used. Each tube was inoculated with the yeast and observed daily for acid production and gas formation.

Sugar assimilation. The auxanographic method of Lodder and Kreger-van Rij (ibid) was used.

Capsule formation. Smears of yeasts were prepared and treated with Indian ink to show the presence of capsules.

Production of spores. The yeasts were streaked on Gorodkowa agar (Lodder and Kreger-van Rij, ibid) and the plates

incubated upside down. The production of ballistospores was evident by the presence of spores on the lid of the dish. Ascospores were seen by microscopic examination.

Morphology. Cultures were grown on Difco Yeast morphology agar and examined after seven days.

Identification of moulds

These were identified to generic level on the basis of the structure of their fruiting bodies (Barron, 1968; Barnett, 1955).

CHAPTER THREE

MICRO-ORGANISMS IN THE PHYLLOPLANE

EXPERIMENTAL DESIGN

The primary factors affecting the presence or absence of specific organisms on the leaf surface are presumably the nutrient status and pH of the substrate, the available moisture and the temperature.

In examining the saprophytic microflora of growing trees, the following variables were examined to give as wide a consideration as possible of the effect on the phylloplane microflora of changes in these primary factors.

In each case care was taken to reduce the number of variables to a minimum. All samples were collected at approximately 9 a.m. in fine weather. For general sampling a mixture of current and second year needles was taken from the shaded aspect near the bottom of three eight to ten-year-old trees at Bottle Lake plantation near Christchurch. Changes in this procedure were made only where needed to test a particular variable.

Position on the tree

In order to ascertain the vertical distribution of microorganisms on the trees, samples were taken from both top and bottom branches.

Aspect of the tree

A comparison was made of numbers and types of organisms living on the shaded and sunny aspects of the tree. Care was taken to select trees with distinct sunny and shaded aspects.

Age of needles

The populations of epiphytic micro-organsims were determined on 6-month-old needles, 18-month-old needles and 30-month-old needles.

Age of tree

Bacteria, yeasts and moulds were isolated from trees in each of two compartments at Bottle Lake. The first compartment contained trees all between seven and nine years old, while the second consisted of stands of 30 years or more.

Season

Sampling was carried out on five occasions between June 1967 and May 1968. Needles from three trees were taken on each date.

Locality

The phylloplane microflora of three trees from Bottle Lake was compared with that from three trees from Kaiangaroa State Forest and from Reefton State Forest. The Kaiangaroa trees selected were from compartment 1205 consisting of trees approximately eight years old. The Reefton trees were on a hilly section and again were approximately eight years old.

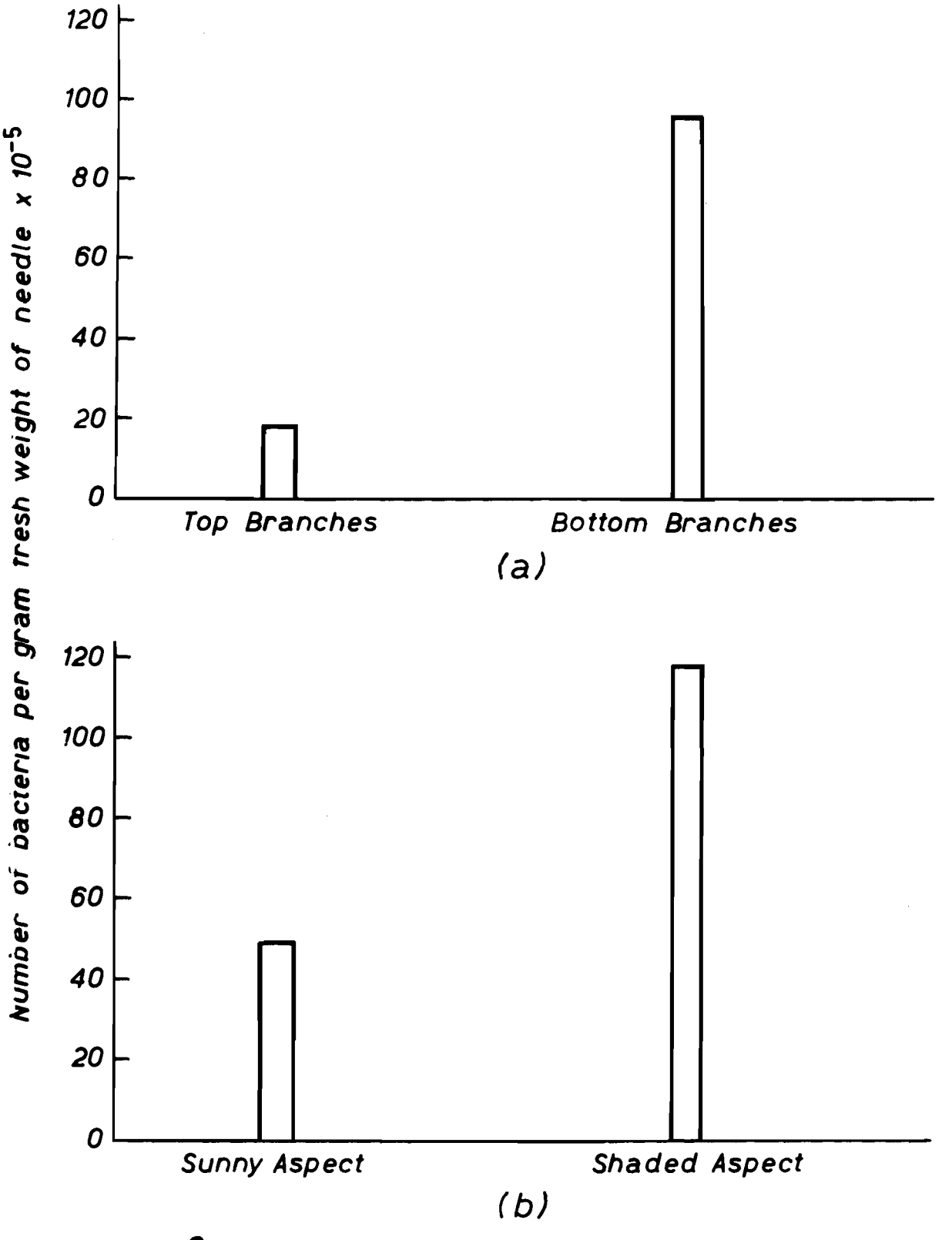


Fig. 2. Variation of total bacterial numbers with position on tree.

BACTERIA IN THE PHYLLOPLANE

Numbers of bacteria

Figs 2-6 summarize the total numbers of bacteria isolated during the six surveys of the epiphytic microflora of Pinus radiata needles. The number of bacteria varied between 10^6 and 10^7 per gram fresh weight of needles. The surface area for one sample of 30 needles was calculated as 500 sq mm $\pm$ 20 sq mm. On this basis there was an average of 4×10^3 bacteria per sq mm of needles if the bacteria were evenly distributed over the needle surface.

Large increases in total numbers occurred from the top to the bottom of the tree (Fig. 2a) and from the sunny to shaded aspect (Fig. 2b). Smaller increases in total numbers were seen with increasing age of needles (Fig.4).

When the epiphytic microflora of needles from eight-year-old trees was compared with that of needles from a stand over thirty years old very little difference in total numbers was seen (Fig. 3). Neither was there any difference in total bacterial numbers when trees from different localities were examined (Fig. 6). There was however a seasonal fluctuation in total numbers. Figure 5 shows a marked increase in total numbers in Canterbury in early Spring, dropping again in late Summer and continuing at a low level throughout the Winter.

Kinds of bacteria

Fifty bacteria were isolated from each sample; about 2,000 were tested using the appropriate tests described in Chapter 2. On the basis of these tests the bacteria were

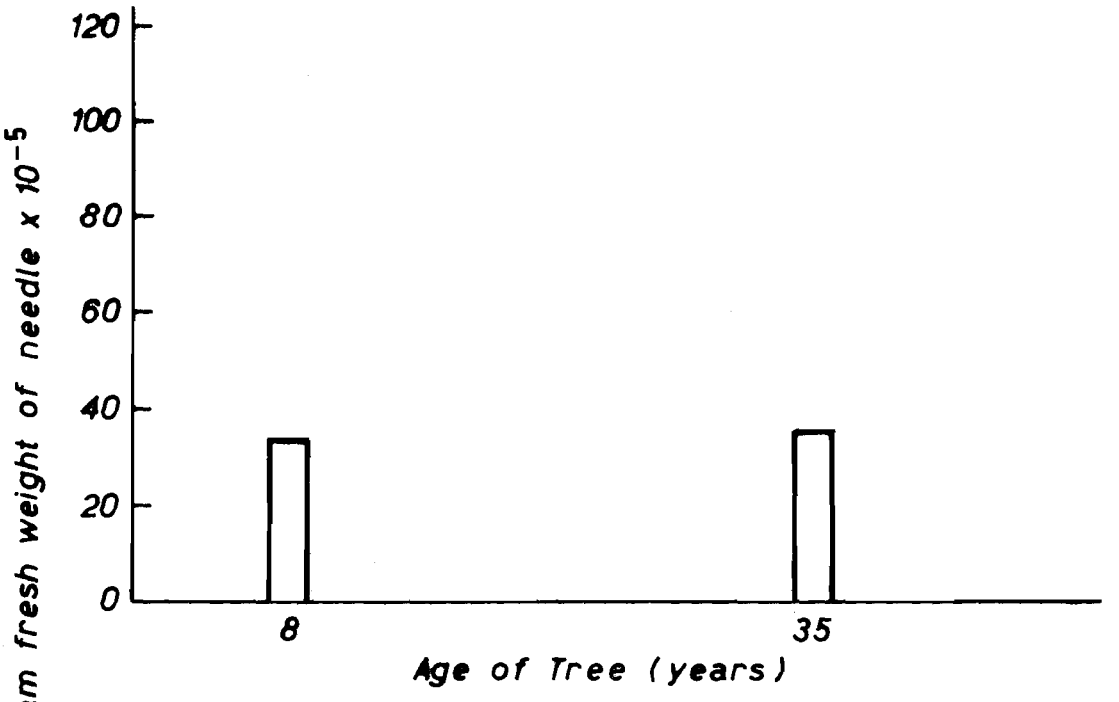


Fig. 3 : Variation in total bacterial numbers with age of tree.

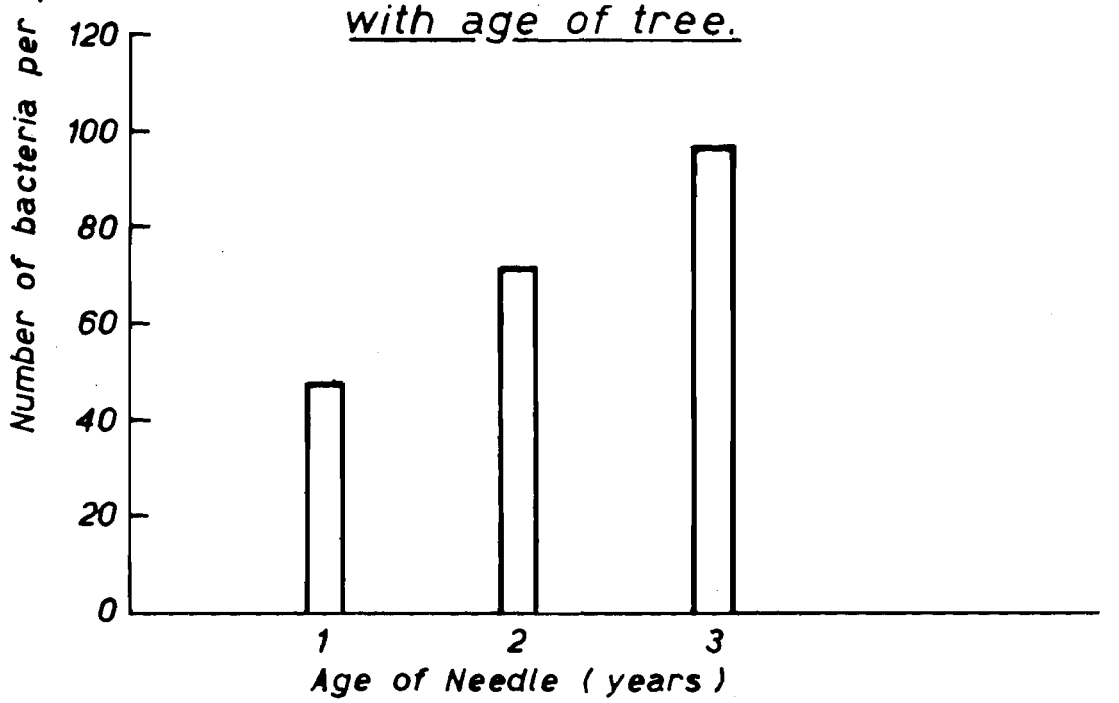


Fig. 4 : Variation in total bacterial numbers with age of needle.

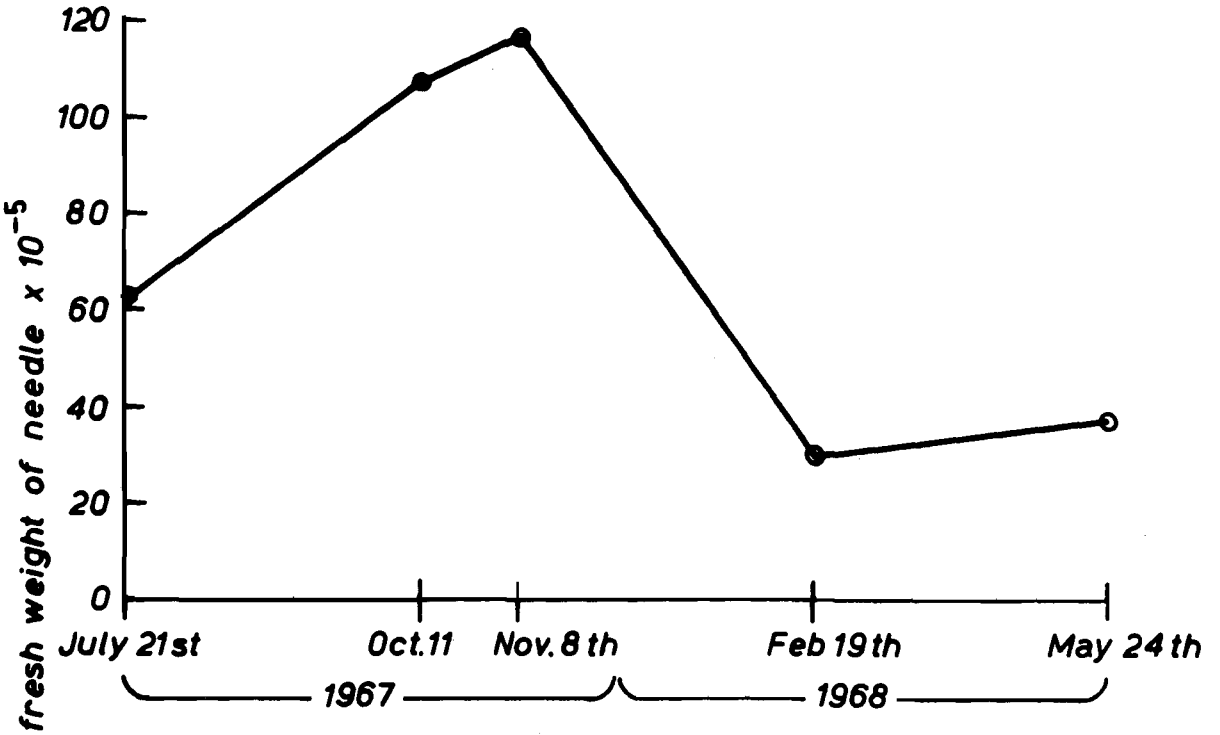


Fig. 5. Variation in total bacterial numbers throughout the year

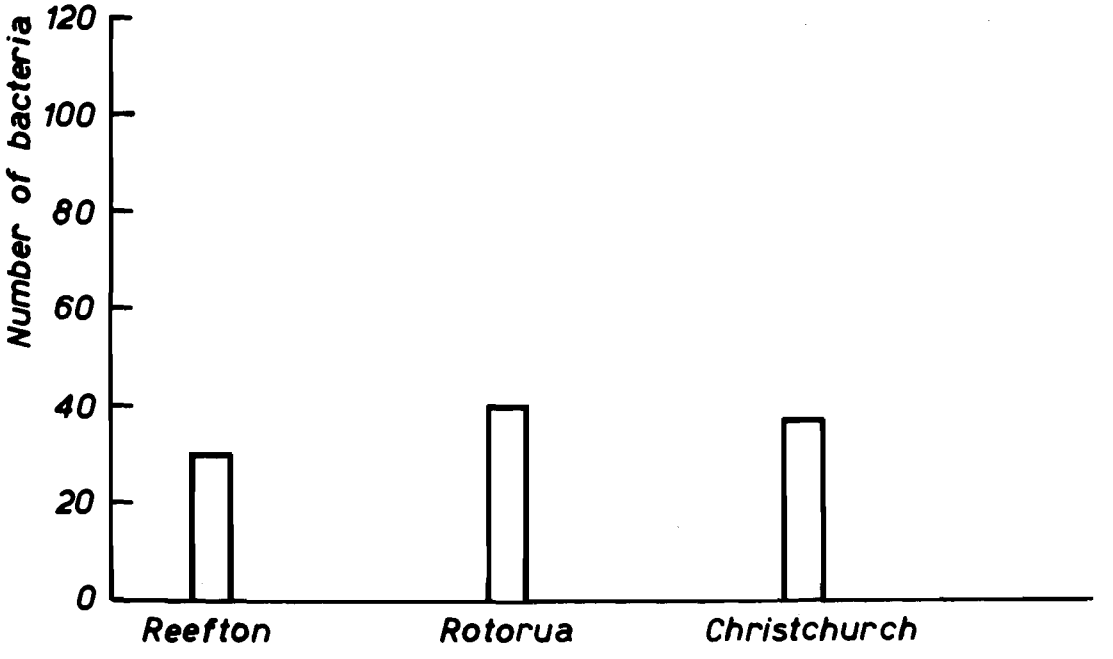
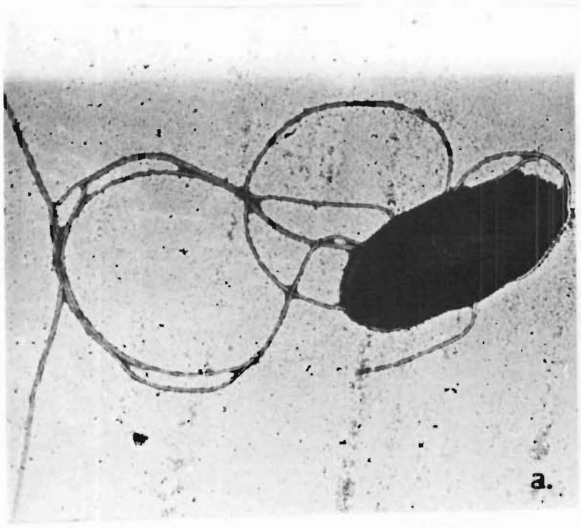


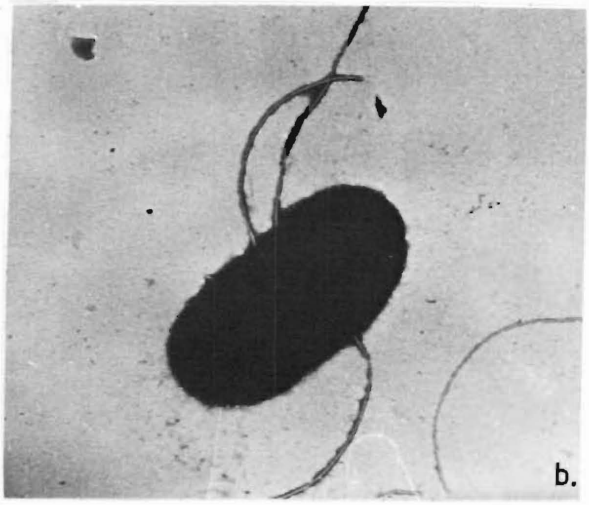
Fig. 6. Variation in total bacterial numbers with locality

placed in four broad groups. These groups conformed to existing taxonomic groups and also reflected the activity of the different kinds of bacteria. The detailed results are shown in Appendix I.

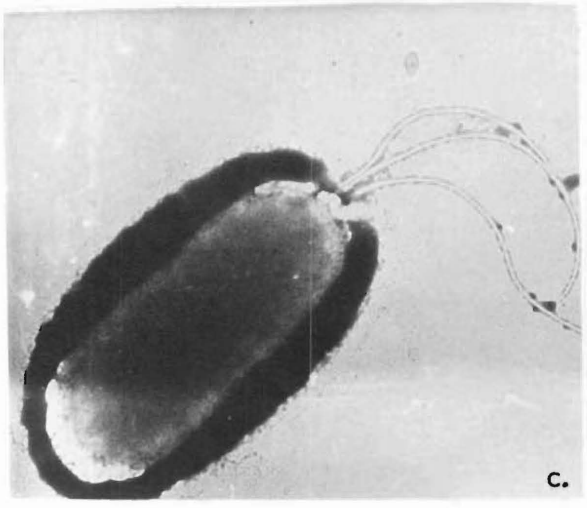
Group A. Coryneform bacteria. This group consisted of Gram-positive or Gram-variable rods, the majority of which exhibited the characteristic angle snapping or pallisade arrangement of the cells. They were either non-motile or only some cells were motile. Colonies varied in colour from white to cream, yellow, orange or pink. In all cases fermentation of glucose was either negligible or very slow. All isolates produced the enzyme catalase. Other tests showed the bacteria to be variable in the metabolism of the carbohydrates lactose and sucrose although the reaction was always slow if it occurred. Few cultures were tolerant of NaCl at a concentration of 10%. Liquefaction of gelatin and conversion of nitrates to nitrites was variable throughout this group. Reaction in litmus milk also varied. This is a heterogenous group, some members appearing very like some flavobacteria except in Gram reaction, while others were clearly Arthrobacter. None were acid fast, which rules out the presence of Mycobacterium.

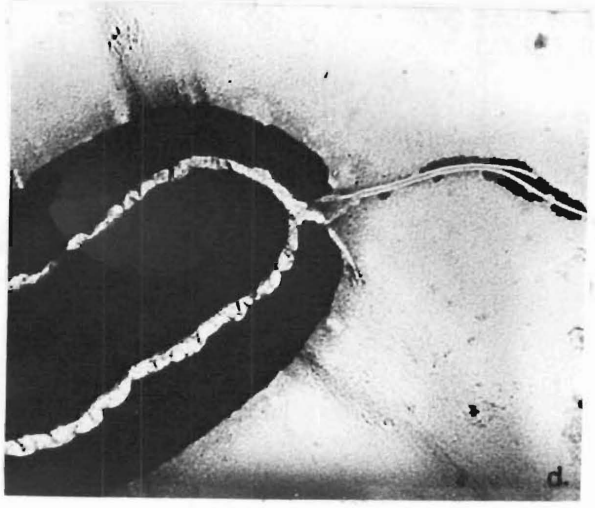
Group B. Lactic acid bacteria. These were similar to the above but did not produce the enzyme catalase, nor did any isolates exhibit angle snapping or pallisade arrangement of cells. This aligned them with the lactic acid bacteria according to Breed et al. (1957) and to the scheme of Harrigan and McCance (1966). The majority were bacilli but





Peritrichous flagella. x 20,000.





Polar flagella. x 36,000.

Fig. 7 Electron Micrographs of Bacterial Flagellation.

there were also some coccoid forms (about 5%). Cultures in all cases were non-pigmented.

Group C. Gram-negative bacteria. These were further grouped as follows:

(i) Pseudomonads. Bacteria placed in this group were mainly non-pigmented although a few yellow and pink forms occurred. They were motile short rods with polar flagella (Fig. 7 c, d). Most produced fluorescin on Medium B of King et al. (1954). All utilized glucose oxidatively and produced the enzyme oxidase extracellularly. Most cultures peptonized litmus milk and liquefied gelatin.

(ii) Paracolons. The other non-pigmented rods were coliforms according to the scheme of Hendrie et al. (1968) and Harrigan and McCance (1966). However since these organisms did not come from the colon the term paracolon is used (Shewan et al., 1960). These differed from the pseudomonads as they utilized glucose fermentatively.

(iii) Erwinia group. Some yellow bacteria conformed to the characteristics given by Hendrie et al. (ibid) and Harrigan and McCance (ibid) for the Erwinia group. These bacteria fermented glucose producing acid, and were motile, often clumping together in the medium. They did not produce oxidase. Flagellation was peritrichous in the 20 cultures examined (Fig. 7 a, b) and 60% were tolerant of a 10% NaCl concentration. The majority produced acid in litmus

milk and liquefied gelatin, although in some cases the latter reaction took up to fourteen days.

(iv) Flavobacterium group. The remainder of the pigmented bacteria and a few non-pigmented types were classified in this group. They were all motile short rods, producing the enzyme oxidase and most liquefied gelatin. In most cases the production of acid from glucose was very slow and weak. Reaction in litmus milk was variable as was production of acid from lactose and sucrose.

Group D. Cocci. All the cocci isolated excepting those placed in Group B were included in this group. They were either unpigmented or yellow. All were Gram-positive, non-motile and utilized glucose fermentatively. Most of these bacteria were slow in their reaction to tests.

Relative proportions of bacterial groups

The relative proportion of the different groups of bacteria was determined for each sample. The plant coryneform group was dominant in all cases, usually accounting for over 50% of the total bacteria present. The proportion of Gram-negative bacteria varied from approximately 7% to over 28% of the total and lactic acid bacteria from 6.5% to approximately 28%. In some cases cocci were absent, in others they comprised more than 7% of the bacteria present.

The variations in proportions of these groups with different conditions are represented in Table VI. Examination

Table VI. Relative proportions of kinds of bacteria isolated

Source	% of Total Numbers Isolated			
	Coryneform	Lactic Acid	Gram-ve	Cocci
Top of tree	71.0	17.0	12.0	0.0
Bottom of tree	69.6	13.6	16.8	0.0
Shaded Aspect	60.1	12.4	24.0	3.5
Sunny Aspect	77.0	10.8	7.6	4.6
1st yr needles	77.8	11.1	11.1	0.0
2nd yr needles	71.7	6.5	19.0	2.8
3rd yr needles	63.3	10.0	24.6	2.1
Trees under 10 y	70.5	7.1	20.3	2.1
Trees over 30 y	60.0	13.0	24.0	3.0
Canterbury	50.0	20.2	24.7	5.1
Rotorua	39.2	28.3	25.2	7.3
Reefton	50.0	19.6	28.2	2.2
June	70.8	13.2	14.0	2.0
July	72.7	10.8	15.0	1.5
November	60.1	13.8	23.0	3.1
February	60.0	15.0	23.0	2.0
May	51.0	20.2	23.7	5.1

of these figures shows that the kinds of bacteria present did not change within the conditions tested. There was some variation however in the relative proportions of the groups present. Gram-negative bacteria were present in larger numbers on the shaded aspect of the tree than where needles were exposed to sunlight. Lactic acid bacteria appeared more numerous on trees over thirty years old than on those under ten years old. They were also more prevelent on trees grown in Rotorua. On the other hand trees grown in Rotorua appeared to support a slightly smaller proportion of coryneform bacteria than trees from either Canterbury or Reefton.

Some seasonal fluctuations may occur. The coryneform population decreased from July to the following May while over the same period there was a parallel increase in both lactic acid bacteria and Gram-negative rods.

The numbers of cocci are too low to show significant variation.

It was also possible that some kinds of bacteria within these groups might fluctuate even more with the environment. For this reason the possibility of further subdividing the groups was investigated. Various schemes for separating the plant and soil coryneform bacteria have been used (Clark *et al.*, 1951; Jensen, 1952; Gibson, 1953; Keddie *et al.*, 1966; Robinson, 1968; Allen, 1970). Most of these require a larger number of tests than was possible within the scope of this study so no valid basis could be found for further subdivision of this group. The Gram-negative bacteria were sub-divided as described previously and their distributions determined. However since the numbers isolated from each sample were so small no pattern of distribution could be found on the basis

of those isolated. The average numbers and types of Gram-negative bacteria isolated from each sample are given in Appendix III. Because of their specialized nature and infrequent occurrence the lactic acid bacteria and the cocci continued to be regarded as single groups.

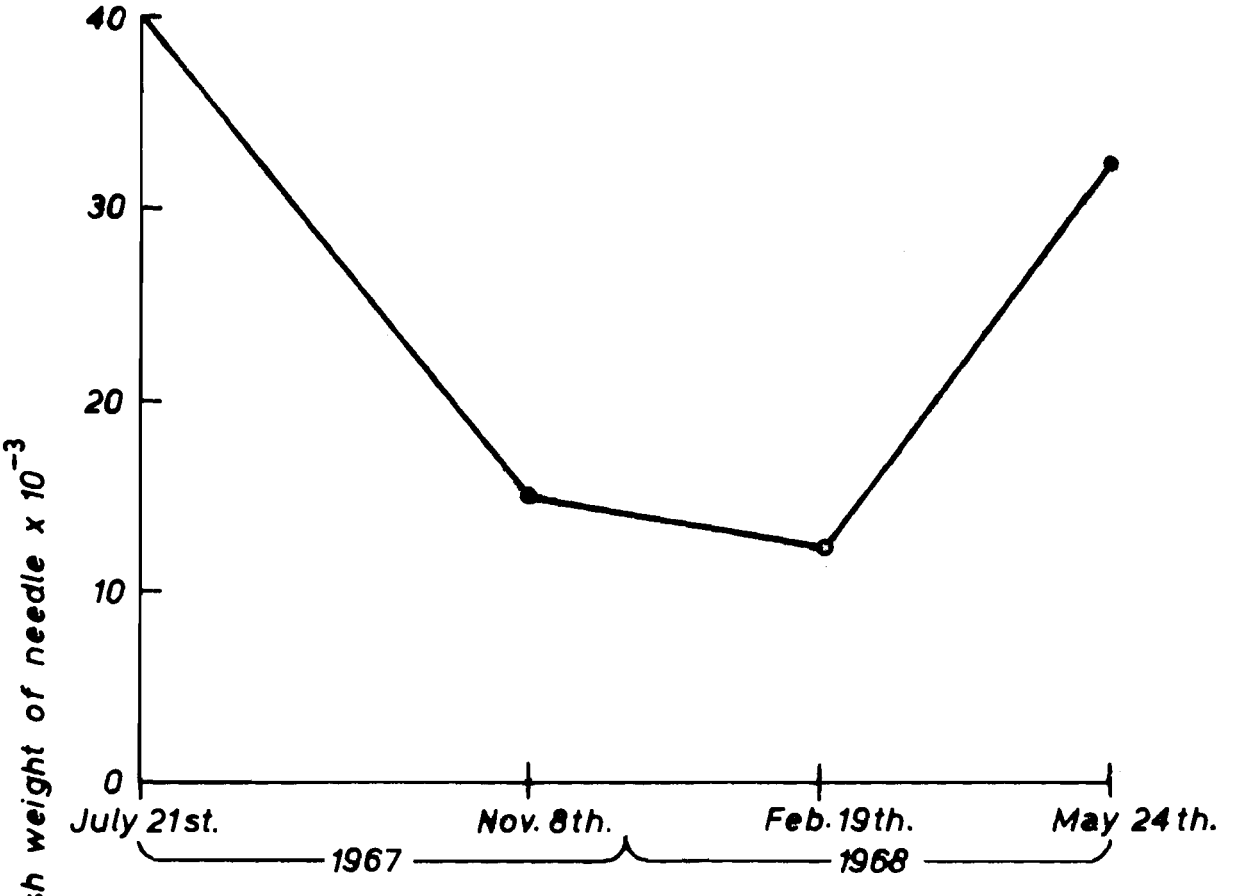
YEASTS IN THE PHYLLOPLANE

The number of yeasts per gram fresh weight of needles varied between 10^2 and 10^4. Because of early difficulties in distinguishing yeasts from some of the bacteria insufficient samples were obtained to show their variation under different conditions.

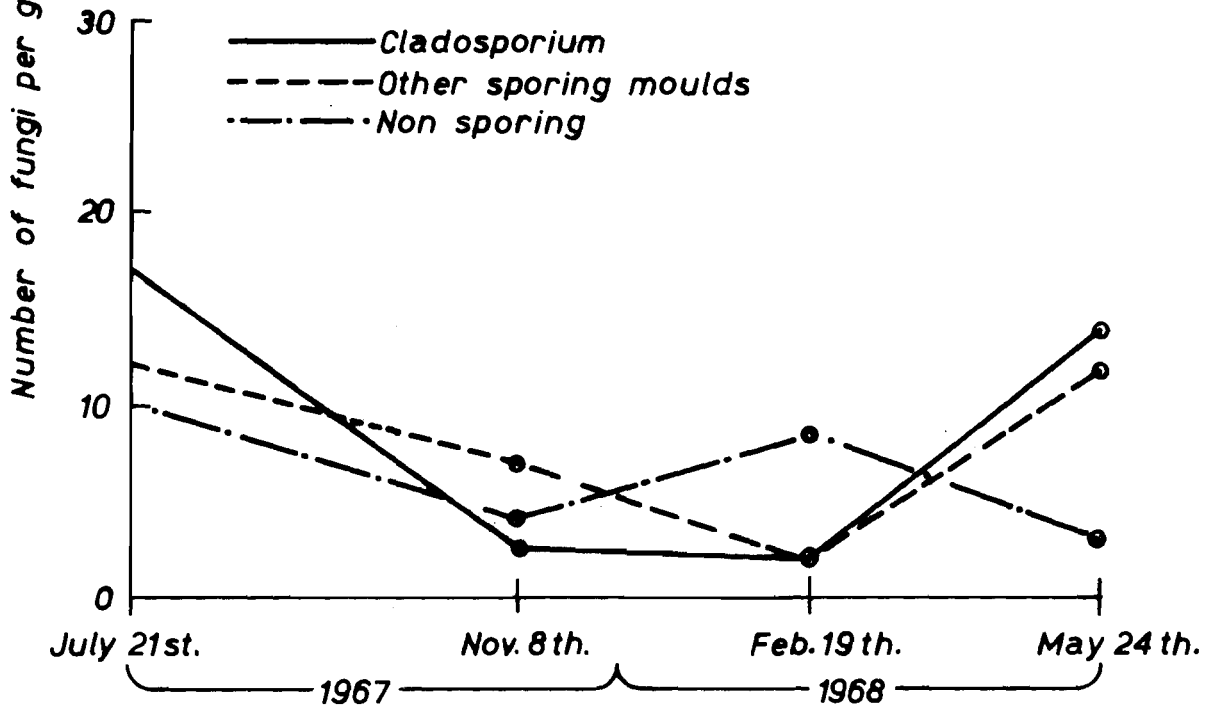
The kinds of yeasts most commonly observed however were as follows: Cryptococcus 34% of the total isolated, Torulopsis 26%, Rhodotorula and Sporobolomyces 4%. Other genera isolated have been identified as Hanseniospora and Bullera. Also present in low numbers was the yeast-like mould Aureobasidium pullulans. About 20% of the yeasts isolated were not identified by the tests used. Some of these formed pseudomycelium on malt extract agar. These however were of sporadic occurrence in low numbers.

MOULDS IN THE PHYLLOPLANE

In all cases the number of moulds isolated was of the order of 10^4 per gram fresh weight of needles. The most frequently isolated genus was Cladosporium which accounted for between 20% and 50% of the total count. Penicillium spp. were usually present although often in very low numbers:



(a) Variation in total number



(b) Variation in main groups of moulds

Fig. 8 : Seasonal variations in Saprophytic moulds.

2% to 8% of the total. Other genera isolated with some regularity included: Cephalosporium, Alternaria, Aspergillus, Pestalotia, Botrytis, Stemphylium, Ramularia, Trichoderma and some members of the order Mucorales. Many filamentous fungi isolated did not produce spores in culture and have been grouped as "non-sporing moulds". Further identification of these fungi is an extension of this work which could be valuable.

A great diversity in the occurrence and proportions of these genera was noted even within a single sample. It was common to find two trees in the same sample on which occurred different numbers or genera of moulds. Thus no significant difference could be found in either total numbers or in relative proportions of fungi present under the conditions tested.

The results shown in Fig. 8a indicate a large drop in numbers over the summer months. Examination of Fig. 8b suggests that the decrease in numbers is brought about by a decrease in the numbers of spore producing moulds and of Cladosporium in particular. The numbers of non-sporing moulds fluctuated slightly throughout the year. The apparent rise in numbers of these in February could be due to low counts of sporing fungi allowing either a greater recovery of non-sporing isolates or an increased growth of these fungi on the needle.

DISCUSSION

This survey revealed the presence of an epiphytic microflora on the surface of Pinus radiata needles and pointed out some of the environmental factors influencing this microflora.

Numbers of bacteria

The estimated number of bacteria varied between 10^6 and 10^7 per gram fresh weight of needles. This count is similar to that found by several other workers. Burri (1903) and Düggeli (1904) macerated leaves of members of the Gramineae, Leguminosae and Compositae and counted between 10^5 and 10^7 bacteria per gram fresh weight of leaf. Hislop and Cox (1969) recovered 10^8 cells per gram fresh weight from washed and macerated apple leaves. Stout (1960) found a greater variation when he isolated 10^4 - 10^{12} bacteria per gram fresh weight of leaf of both ryegrass and white clover and Gibson et al. (1958) also found a variation in numbers of bacteria on ryegrass. They isolated 10^6 - 10^8 bacteria per gram dry weight from perennial ryegrass.

Position on the tree

The same environmental conditions were probably responsible for the fact that greater numbers of bacteria were found on needles near the bottom of trees than on needles from the top, and more from needles on shady aspects than from those in sunny positions. The uppermost branches are exposed to more extreme conditions of wind, sunlight, rain and humidity than the more sheltered lower branches. Needles on lower branches also collect nutrients leached from above (Tukey, 1966). For a tree to have a distinct "sunny side" there must be a gap in the canopy of the forest adjacent to that tree. Because of this, not only is that side of the tree exposed to more sunlight, but also to more wind and rain.

Age of needle

The build up in numbers of bacteria on the needle surface as the needles age could result from a summation of capture of air-borne bacteria over the three years. This does not seem to be the case however as when extra bacteria were sprayed on the younger needles numbers did not increase (see Chapter 5). The increase in numbers on older needles must therefore be related to the ability of the older needles to support an increased population of bacteria. Tukey (1966) has shown that exudation of nutrients from leaves of a variety of plants increased as the needles aged, reaching a maximum just prior to senescence.

Age of tree

It appears that it is the age of the needle rather than that of the tree which is important in determining the numbers of bacteria supported on the needle surface. Since needles of P. radiata are replensihed every three years the needles examined on trees 8-years-old and 30-years-old were of comparable age, and no significant difference between their microfloras could be found.

Seasonal variations

An attempt was made to correlate the fluctuations in total numbers throughout the year with graphs showing changes in climate in Christchurch. Fig. 9 shows monthly variations in rainfall and temperature throughout the period of sampling. Fig. 10 gives the average humidity and temperature for the 24 h prior to sampling. Wind velocity and rainfall at the time of sampling were negligible. These figures were obtained

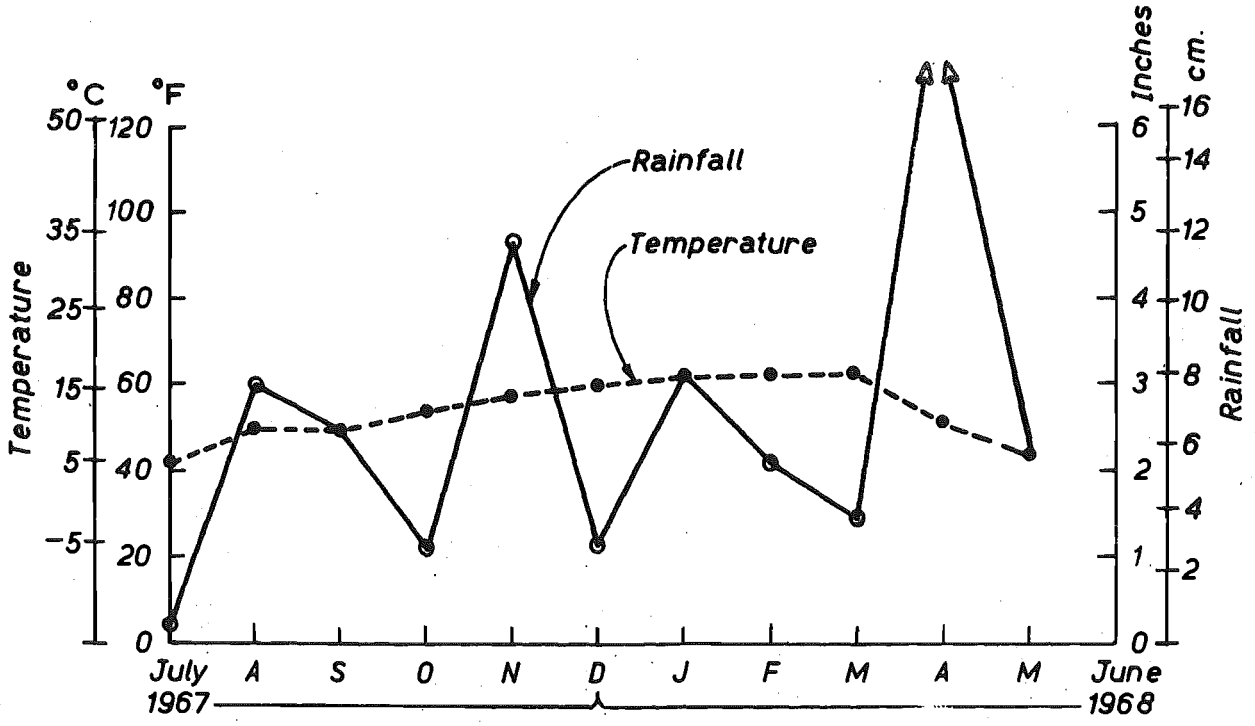


Fig. 9 Variation in mean monthly rainfall & temperature throughout period of sampling

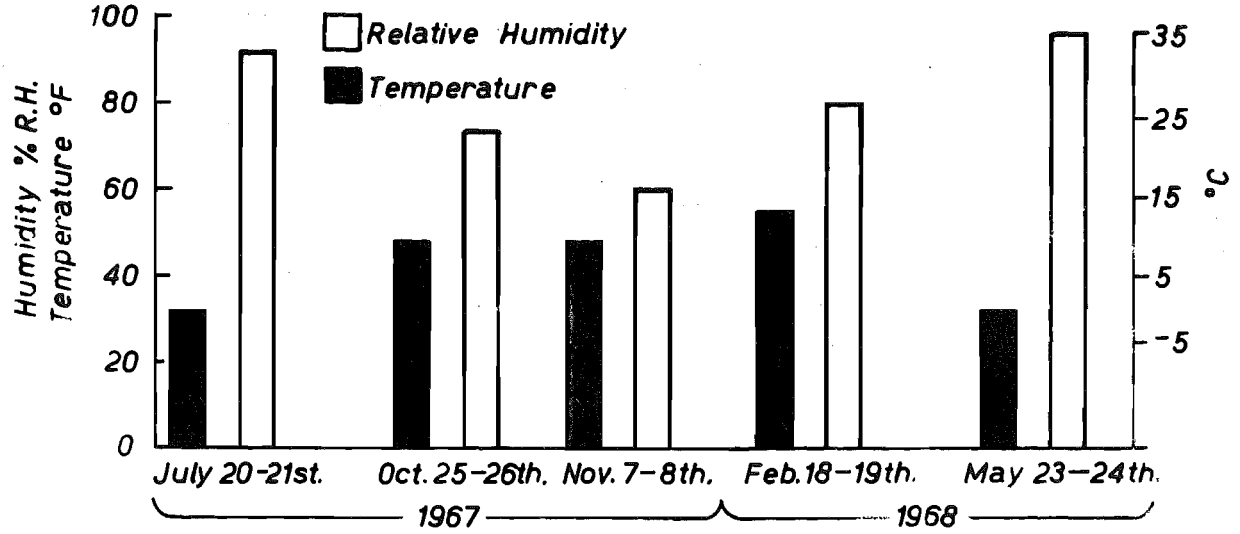


Fig. 10 Mean temperature & relative humidity over 24 hour period prior to each sampling

from the meteorological service at Christchurch Airport and, while not accurate for Bottle Lake approximately 8 km away, they do give an idea of fluctuations in the climatic conditions in the general locality. Comparison of these graphs with the curve obtained for bacterial numbers makes it obvious that the changes in temperature, humidity and rainfall could not directly account for the increase in bacterial numbers from July to November. It is more likely that this peak corresponds to the increased rate of growth of the tree occurring at this time. Experiments on the effect of varying temperature and humidity on the microflora of seedlings are reported in Chapter 7. The influence of needle leachate on bacterial numbers is also described.

Locality

In view of the very different localities examined it seemed surprising that the total numbers of bacteria found were not significantly different. However the high bacterial numbers could be correlated with increasing age of needles and with periods of rapid growth rather than with seasonal variations in temperature and rainfall. This suggests that temperature and moisture might not be as important in determining the size of the population on needles within the canopy as factors governed by the host tree. If this is so then little variation in total numbers would be expected. This hypothesis is supported by work reported in Chapter 7.

Kinds of bacteria

Comparison of the results obtained by various workers has been complicated by their use of different systems of identification, but there is a general agreement that the populations of leaf bacteria are distinguished by a large proportion of pigmented isolates (Stout, 1960; Vosnyakovskaya and Khudyakov, 1960; Last and Deighton, 1965). Stout (ibid) suggested that since few pigmented forms occur in the soil pigmentation might be an adaptation against the harmful effects of u.v. radiation in a habitat exposed to sunlight.

In the various samples examined in this survey between 25%and 40% of the bacteria isolated were pigmented. This is a higher proportion than that quoted by Stout (1960) for soil bacteria, but it does not reach the maximum proportions which he gives for his phylloplane isolates (95%). If resistance to u.v. irradiation is indeed selective for pigmented organisms it is possible that bacteria on pine needles within the forest canopy have less need of pigmentation than those in a grassland environment. The lack of change in proportions of pigmented bacteria from sunny to shaded aspects of the tree (Appendix IV) suggests that irradiation within the canopy is not of great importance.

Both Gram-positive and Gram-negative bacteria have been found on leaf surfaces, although the relative abundance of the two groups varies with the different studies. The present survey indicated that 60 - 80% of the bacteria on Pinus radiata needles were Gram-positive coryneform rods and lactic acid bacteria. Gram-negative rods (flavobacteria, pseudomonads, paracolons and Erwinia) accounted for most of the remainder,

while micrococci were usually present in low numbers. These results are similar to those obtained by Gibson *et al.* (1958) who noted that many of the bacteria isolated from freshly cut ryegrass were Gram-positive and coryneform. They also noted an infrequent occurrence of lactobacilli. Lactic acid bacteria have been reported on grass leaves by Keddie (1951), Kroulik *et al.* (1955) and Nilsson and Nilsson (1956). In 1969 Hislop and Cox, while not fully characterizing their isolates, noted that both Gram-positive and Gram-negative bacteria occurred on apple leaves.

In contrast to these results are the early reports of Burri (1903) and Düggeli (1904) who found *Erwinia herbicola* and *Pseudomonas fluorescens* to be dominant bacteria on a wide range of plants. It might be argued that the use of more varied techniques by later workers allowed the isolation of a greater range of bacteria, but some recent reports also show Gram-negative rods to be more numerous than Gram-positive bacteria. Stout (1960) found *Flavobacterium* to be the most common bacterium on clover and ryegrass pastures in New Zealand, with *Aerobacter*, *Pseudomonas*, *Xanthomonas* and *Micrococcus* less common. Vosnyakovskaya and Khudyakov(1960) by washing leaves of 18 plant species identified *Pseudomonas* as the most common bacterium, followed by *Mycobacterium*, *Micrococcus*, *Bacterium*, *Chromobacterium*, *Pseudobacterium*, *Lactobacillus*, *Bacillus* and *Sarcina*.

These differences are unlikely to be the result of host differences since Vosnyakovskaya and Khudyakov (1960) using a variety of media found no significant differences in the kinds of bacteria isolated from the 18 different species which included cereals, pasture plants, fruit trees and pine. This

suggests a lack of host specificity. Also Gibson et al. (1958) isolated a predominantly coryneform group from ryegrass, while Stout (1960) also working on ryegrass showed Flavobacterium to be the most important genus and found no coryneforms. In most cases although similar groups of bacteria have been isolated from the leaf surface by different workers, the relative abundance of each group has varied. Most of Stout's Gram-negative bacteria were isolated from P. radiata but in very low numbers, while the bacterial population Gibson et al. (1958) found on ryegrass leaves is very similar to that found on P. radiata in the present study. It is possible that in some studies the plates were not kept long enough to allow the isolation of the plant coryneform group. In some cases these took two weeks to appear on plates.

Although bacterial populations varied in size on needles in different positions on the tree (Fig. 2) the kinds of bacteria present did not (Table VI). This suggests a relationship between the saprophytic microflora and the host tree in which the host largely determines the kind of saprophytes present. Tukey (1966) has shown that leaching of nutrients from leaves of plants is a universal occurrence.

> " Inorganic nutrients leached, include all of the essential nutrients and some other elements commonly found in plants.... Large amounts of carbohydrates can be leached including the free sugars fructose, glucose, raffinose and sucrose, polysaccharides and pectic substances.... All the 21 amino acids and derivatives found in plants can be leached to varying degrees and large amounts of organic acids are also detected in the leachates. "

Obviously this leachate provides a wide range of nutrients that may be utilized by micro-organisms on the leaf surface and factors affecting exudation would of necessity

influence the population occurring there. It appears that under the conditions tested the composition of the metabolites leached from the leaf surface did not vary enough to significantly alter the kinds of bacteria present. Changes in total numbers may however, be correlated with changes in the amount of leachate.

Yeasts on the needle surface

No data were obtained on the distribution of yeasts on the tree. However some of the common types were identified and were found to be similar to those obtained by di Menna (1959) from the leaves of pasture plants and by Ruinen (1963) working on tropical plants. On pasture plants in New Zealand di Menna showed an increase in the red pigmented Rhodotorula and Sporobolomyces spp. in late summer and early winter. No such fluctuations occurred in numbers of yeasts inhabiting the needle surface of Pinus radiata. The types present varied only slightly throughout the year, there being often greater variations within samples than between samples. Last (1955) suggested that an important factor governing the seasonal incidence of Sporobolomyces on cereal leaves might be the age of the leaf. He suggested that Sporobolomyces colonizes the leaf towards the end of its life - possibly because some essential nutrient is released with approaching senescence. If this is so it is possible that similar factors account for the low numbers of Sporobolomyces in the present survey. In all cases yeasts from current year's needles were examined. The fact that these needles live for three years rather than just one season may account to some extent for the lack of seasonal variation in this habitat.

Moulds on the needle surface

The population of moulds in the phylloplane of P. radiata appears less stable than the bacterial population since both numbers and kinds fluctuated greatly. It is difficult to correlate plate counts of filamentous fungi with the actual population present on the needle surface. Such counts do however, show major changes in the total biomass of the moulds isolated.

The fungal genera isolated in this study were similar to those found by Dickinson (1967) on Pisum leaves and Hollomon (1967) on potatoes. Di Menna and Parle (1970) recovered a greater variety of moulds from pasture plants but this could be expected in a habitat where contamination from the soil is easy. Hogg and Hudson (1966) isolated a number of filamentous fungi from the phylloplane of Fagus sylvatica. Some of these fungi were also found in the present study on P. radiata, but the latter isolates were even more similar to the group of fungi isolated by Dickinson (1965) from a very different situation:- leaves of Halimione growing in a salt marsh. One should not attach too much importance to such comparisons as it is possible that differences and similarities are largely the result of the techniques used in isolating the moulds. In all the reports mentioned above however, Cladosporium herbarum was an important component of the population.

The moulds Cladosporium and Penicillium are common components of the air spora in Christchurch (Dye and Vernon, 1952; Peddie, 1963). Most of the other moulds present have also been identified regularly from the air. This is the most likely source of infection for the needles. Early work

in this study showed that stripping the epidermis from the needle removed all the micro-organisms. This suggests that at least on healthy P. radiata they do not invade the needles under the conditions tested.

Considerations of seasonal fluctuations of filamentous fungi on the needle surface however suggest that more than casual contamination of the needles is involved. The data showed a significant drop in total numbers of moulds and of Cladosporium and other sporing types during the summer months. Holloman (1966) showed a similar reduction in fungal numbers on potato leaves but di Menna and Parle (1969) did not find this on ryegrass and clover pastures in New Zealand. Continual cropping and renewal may have been the reason for this.

In contrast to this drop in numbers of moulds on the leaf surface, the concentration of fungal spores in the air increases during the summer months (Dye and Vernon, 1952; di Menna, 1955). If the population on the needle surface was a purely casual contamination then numbers there would be expected to increase accordingly. This does not occur suggesting that factors other than availability of spores govern their presence on the phylloplane. There might be several factors involved, including the increase in bacterial numbers prior to this period, the change in host metabolism as new needles are produced, and the increased temperature over this period (Fig. 9). The effect of temperature has been described in Chapter 7. These factors are possibly all responsible, acting together to change the nutritional and physical environment of the fungus which in turn must modify the habitat. This suggests that the filamentous fungi form an active component of the phylloplane.

CHAPTER FOUR

DISTRIBUTION OF MICRO-ORGANISMS IN THE PHYLLOPLANE

EXPERIMENTAL METHODS AND DESIGN

The previous chapter contained information on the kinds of micro-organisms resident on the needle surface and gave some indication of the factors controlling them. The techniques used however can give no information about the distribution of micro-organisms on this surface, nor do they show whether all needles are colonized in a similar manner.

Previous reports on this aspect give limited information. Observations by Ruinen (1961) showed that bacteria were initially restricted to depressions in the leaves - between epidermal cells and along the veins - in the tropical foliage she investigated. As the population grew it tended to spread out of the depressions in films of water. Fungal hyphae formed a network over the leaf surface. Di Menna (1959) also described yeasts localized in depressions along the edges of epidermal cells of rye grass. In both cases the distribution of organisms over the surface was uneven, some areas carrying higher numbers than others. In most cases groups of bacteria or yeasts appeared to consist of a single species, or more rarely two species forming a mosaic.

This chapter considers the spread of bacteria, yeasts and filamentous fungi on the surface of pine needles.

Microscopic examination

Direct observation by either transmitted or incident light as used by Leben and Daft (1964, 1967) was unsuitable for detection of bacteria or yeasts on the surface of Pinus radiata needles. The small size of the organisms as well as the corrugated surface and small cross section of a pine needle made it impossible to illuminate the surface sufficiently well.

The spread of fungal growth on the surface of the needle was seen by two methods. In both cases needles were collected aged 6 weeks, 12 months and 2 years. Needles prepared for observation by incident light were placed in lactophenol cotton blue for 2 min., blotted dry and examined using a magnification of x200. Photographs illustrating the growth of fungi on the needle surface are shown in Fig. 11.

For study by transmitted light the needles were decolourized in 95% methyl alcohol for 48 h. The needles were then stained by the periodic acid - Schiff technique (Preece, 1959) and examined at magnifications of x100 and x400.

Scanning electron microscope studies

First year needles from 8-year-old trees were cut into 6 mm lengths and mounted on a specimen stub. These were then frozen in freon at - 130°C and freeze dried for 20 h at - 70°C before placing in a high vacuum evaporator and given first a thin coating of carbon and then of gold palladium 0.04 μm thick, while rotating at 150 rev/min.

The prepared specimens were then placed dry in the column of a Cambridge Series II scanning electron microscope for examination.

These studies were carried out at the Physics and

Engineering Laboratory, D.S.I.R., in collaboration with Dr. J. Troughton.

Leaf prints

Leaf prints were obtained by making an impression of the needles on an agar plate. The position of the needle was marked after one hour and the needle removed. The plates were then incubated and later examined for the distribution of bacteria, yeasts and moulds.

Nine leaf prints were made of 6 month, 18 month and 30-month-old needles respectively on plates of soil extract agar, Martin's Rose Bengal- streptomycin medium and glucose peptone agar. These were incubated for 12 days at 25°C and observed daily for the first 5 days, then every 3 days.

A separate set of leaf prints was prepared to separate the Gram-positive and Gram-negative bacteria. Prints of nine 2-year-old needles were made on:

(1) A medium incorporating sodium azide (Packer, 1943).

(2) The medium used by Holding (1960) to select for Gram-negative bacteria.

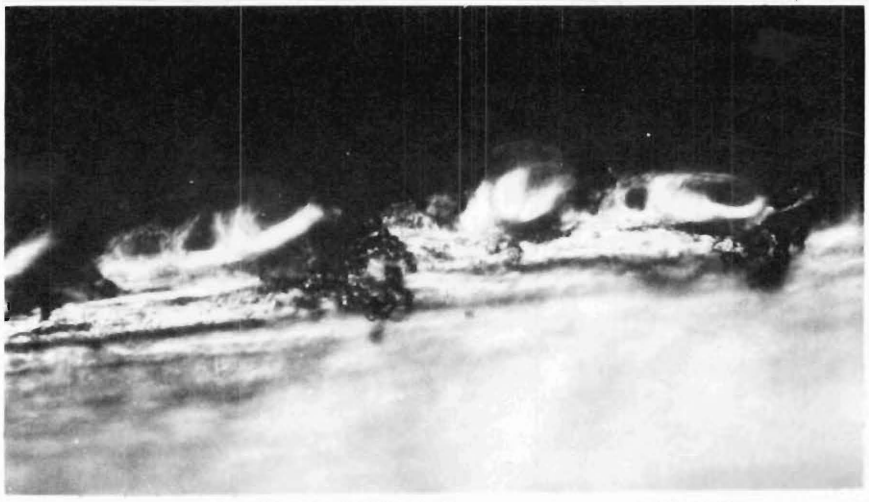
DISTRIBUTION OF MICRO-ORGANISMS IN THE PHYLLOPLANE

Microscopic examination

Examination of the fungi on the needle surface showed colonies to be compact and discrete. The hyphae did not grow profusely over the needle surface. In general fungi were most numerous on the central portion of the needle becoming sparser towards the tip and the base. Fig. 11 shows fungal growth on healthy second year needles. Growth was

) In grooves between epidermal cells.

(b) In the vicinity of stomatal openings.



(c) Beneath projections from the epidermis.

Fungal growth on healthy needles. Fig.11

×400

most common in the vicinity of:

(a) Openings to the stomatal chambers. In a few cases the fungi grew in these chambers (Fig. 11b).

(b) Near the base of small projections from the needle surface (Fig. 11c).

(c) The longitudinal grooves between the epidermal cells on the needle surface (Fig. 11a).

Needles over 12 months old were seen to support a greater density of fungal growth than 2-month-old needles.

Scanning electron microscope studies

The micrographs in Fig. 12 demonstrate the distribution of material on the surface of P. radiata needles. Most of this was confined to the grooves between epidermal cells and to the vicinity of the stomata. It was difficult to distinguish bacteria from other adherents to the leaf such as wax produced by the needles. However some of the structures (Fig. 12a, b, c) resemble in shape and size some of the bacteria seen by Leben and Daft (1969) on soybean buds.

Leaf prints

The leaf prints showed an irregular distribution of bacteria, yeasts and moulds on needles of all ages. Fig. 13 is a diagrammatic representation of the distribution of microorganisms on one set of 2-year-old needles as shown in the leaf prints.

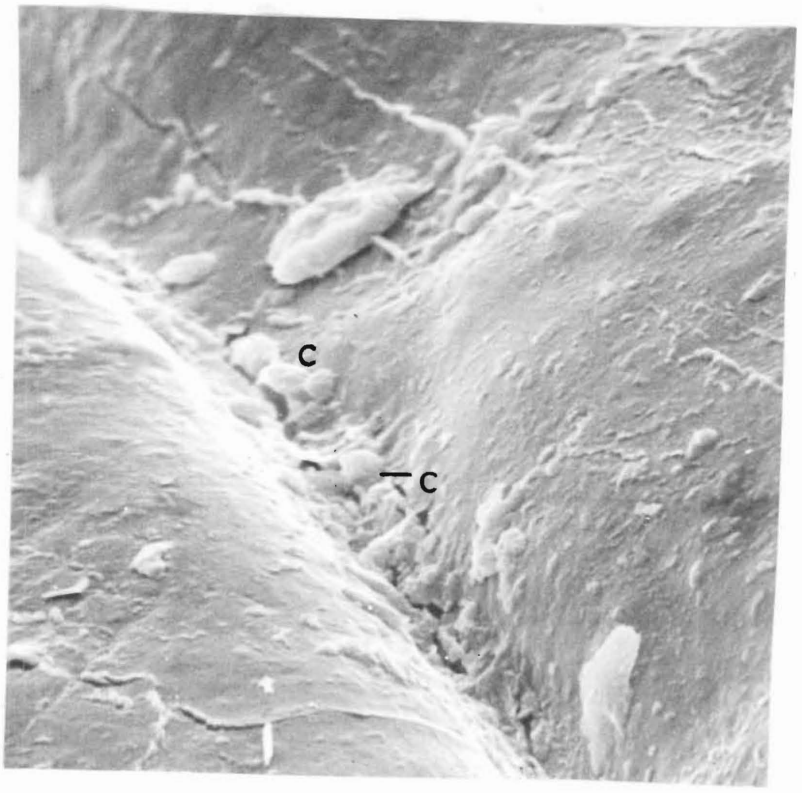
Bacteria. In general bacteria appeared more common on the distal two thirds of the needle. They did occur near the base of 18 out of 27 needles but in smaller numbers than on the

a) Bacteria-like structures in grooves between epidermal cells. x 2700.

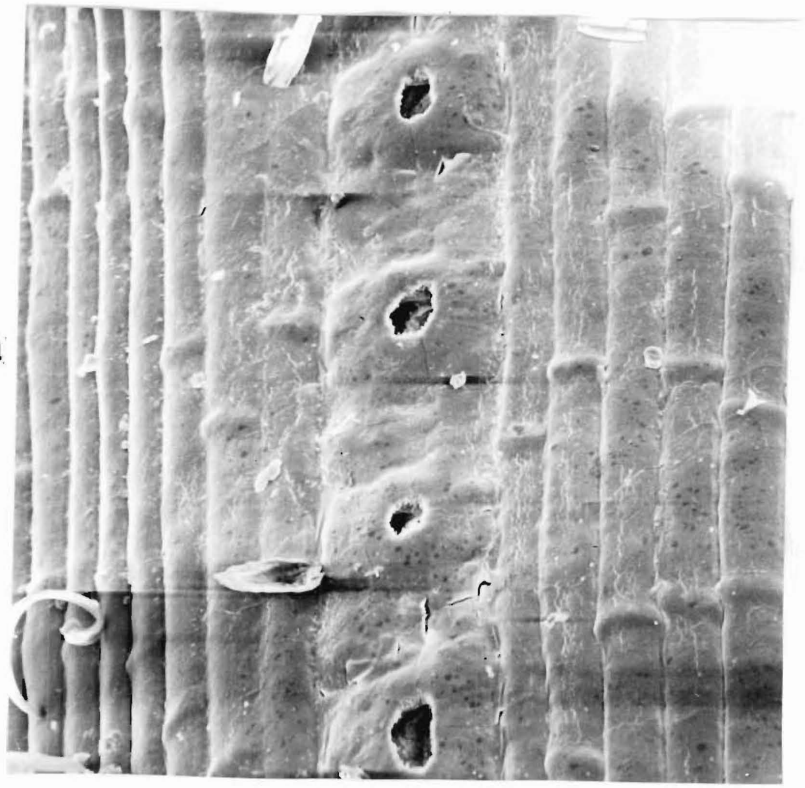
b) Similar structures to a) x 4750.

Fig 12. Distribution of material on the needle surface

c) Bacteria - like structures. x 4750.



d) Surface of P. radiata needle. x 400.



as shown by the Scanning Electron Microscope.

rest of the needles. This was true for needles of all ages. The leaf prints on nutrient agar and on the media selective for Gram-positive and Gram-negative bacteria suggested that most kinds were randomly distributed. From the leaf prints there appeared to be more bacteria on the 3-year-old needles than on 1-year-old.

Yeasts. These occurred irregularly over the entire length of the needles. The genera Cryptococcus and Torulopsis were found on all needles, although their abundance varied from needle to needle. They were not restricted to any area of the needle, occurring as frequently at the base of the needles as on the tip. Rhodotorula and/or Sporobolomyces occurred on 10 out of the 27 needles tested. There was no correlation between the occurrence of these and the age of needles on which they were found. Aureobasidium was also found irregularly and in small numbers on needles of all ages.

Moulds. Moulds were most common on the central portions of the needles, sparser near the tip and least numerous towards the base of the needles. Cladosporium covered the greatest area of the print with Penicillium distributed irregularly down the needles. On some prints isolated colonies of Cephalosporium, Stemphylium, Botrytis, Alternaria and Aspergillus appeared. Non-sporing moulds were not common on the prints but these would be selected against by this technique.

The age of the needles made no difference to the types of moulds isolated from the leaf prints or to the proportions of each type present. However the cover by filamentous fungi was denser on the majority of the 3-year-old needles than on those

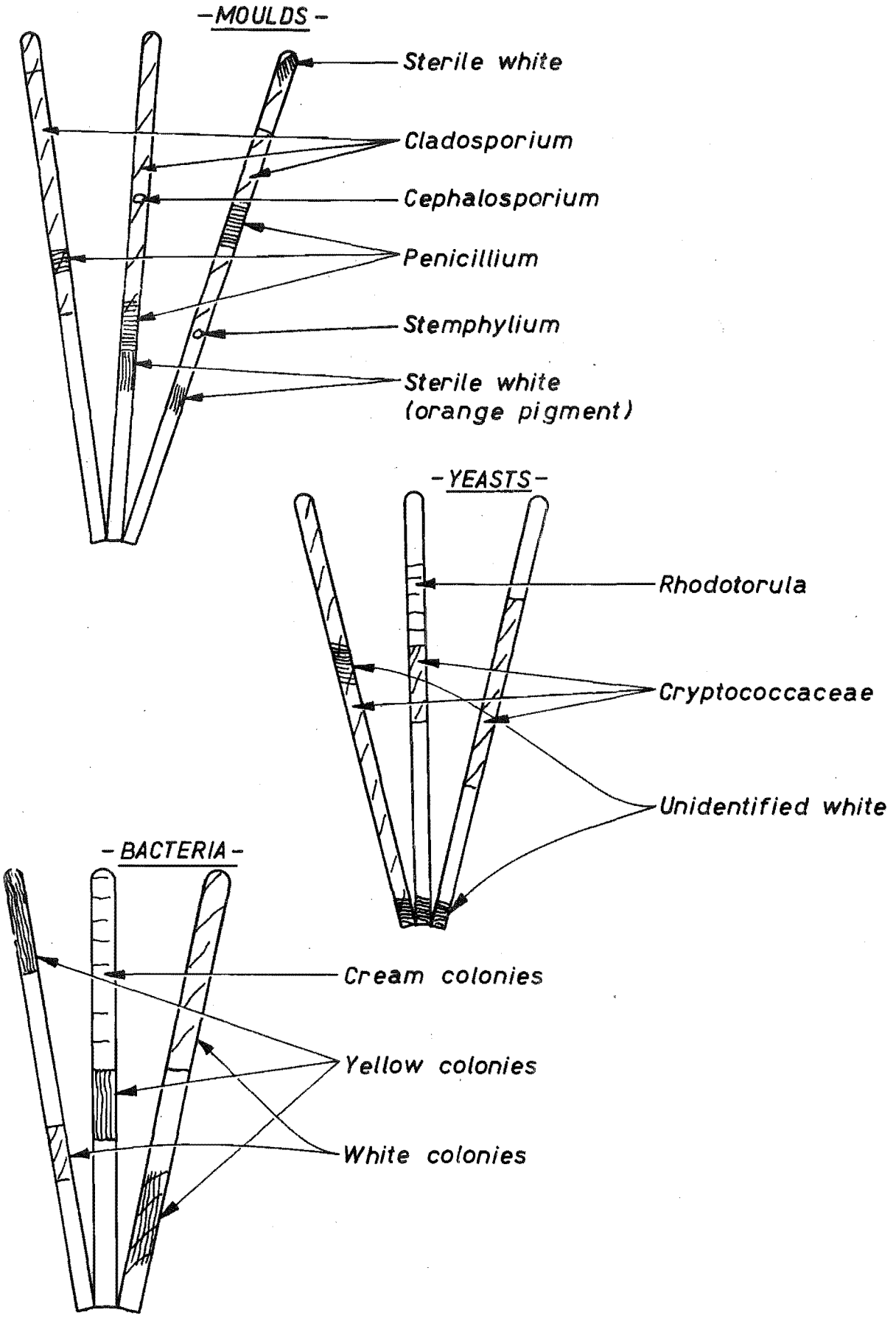


Fig. 13— Distribution of Micro-organisms in leaf prints from 2yr. old needles.

1-year-old. The 2-year-old needles were variable in cover between the extremes shown by both the younger and older needles.

DISCUSSION

Leben (1965) stated that under the microscope many epiphytes are transparent and cannot be seen against the background of the epidermis unless they are stained. He also noted that it was likely that liquids used in staining and making plastic casts of the epidermis might dislodge many organisms. Reports of the spread of micro-organisms on the needle surface must be considered in the light of this. In some cases bacteria have been observed uniformly over the epidermis or in aggregates or colonies (Ruinen, 1961; Simmonds, 1947; Stout, 1960). In other cases, (Last, 1955; Leben and Daft, 1964; di Menna, 1959; Ruinen, 1961; Stout, 1960) bacteria and yeasts have been observed in depressions between leaf epidermal cells. Such a distribution would presumably be affected by the configuration of the leaf surface and by the distribution of water and nutrients.

The corrugated surface and waxy cuticle would cause moisture to run off into the grooves and thus off the Pinus radiata needles. This suggests that the smaller organisms such as bacteria and yeasts may be concentrated around the grooves where moisture and nutrients would tend to remain as the needle dried. These grooves proved difficult to illuminate for study by the light microscope. However the scanning electron microscope with its greater depth of field enables examination of their distribution on the needle surface. The

micrographs (Fig. 12) support the hypothesis that bacteria and yeasts occur almost entirely in the depressions between the epidermal cells.

Direct observation of the fungi suggests that these too are most common in depressions on the needle surface or near the openings to the stomatal chambers where both nutrients and moisture are likely to be concentrated. Fig. 11 shows the fungi to cover only a small proportion of total surface available.

Both leaf prints and direct observation confirmed that populations of microbial epiphytes increased as the needles aged. It was also shown that this increase occurred uniformly over those parts of the needle colonized by the micro-organisms.

The concentration of micro-organisms in grooves between the epidermal cells suggests that nutrients and water are most freely available in those areas. Apart from the base and tip of the needle, colonization is even and non-specific suggesting an even spread of nutrients in those areas colonized by micro-organisms.

This localization of micro-organisms in specialized areas such as grooves between epidermal cells means that although bacteria may be calculated to cover only a small proportion of the needle surface, the cover in those areas suitable for microbial growth is very much higher. In these areas interactions between micro-organisms could be important in determining the distribution and activity of the organisms, including potential pathogens.

CHAPTER FIVE

THE PHYLLOPLANE MICROFLORA AS A POPULATION

INTRODUCTION

It was noted in Chapter 3 that many of the micro-organisms common on the leaf surface of Pinus radiata are also common in either the soil or the air. It may be argued that these micro-organisms are merely casual contaminants and do not constitute a real population. The following experiments were carried out to demonstrate whether the micro-organisms on the needle surface were in fact active in this habitat. The experiments were based on the following hypotheses:

(1) If the micro-organisms were not growing and multiplying in the phylloplane then "recolonization" following surface sterilization would result in a haphazard collection of micro-organisms that need not bear any relationship in numbers or kinds to the original inhabitants. However if the micro-organisms in this habitat constituted a population in dynamic equibrium with themselves and with their host recolonization would follow a recognizable pattern and the final population could bear some relationship to the initial one.

(2) Unless the micro-organisms on the leaf surface were active there would be no reaction to the introduction of new bacteria or fungi into the habitat. If however the micro-organisms were growing and multiplying on the leaf interactions would take

place between the "introduced micro-organisms" and those already occupying the leaf surface.

EXPERIMENTAL DESIGN

In brief, the experimental treatments were as follows:

A Needles of seedlings at Rotorua not surface sterilized.

1. no treatment. (22 seedlings)
2. bacteria from Bottle Lake trees (Christchurch) added. (12 seedlings)
3. fungi from Bottle Lake trees added. (12 seedlings)

B Needles partially surface sterilized.

4. no micro-organisms added. (10 seedlings)
5. bacteria from Bottle Lake trees added. (10 seedlings)
6. fungi from Bottle Lake trees added. (10 seedlings)

METHODS

Seedlings

One-year-old seedlings grown in the Forest Service Nursery at Milton and held in Rotorua for approximately three months were used throughout this study. Shortly after their arrival in Rotorua the young trees were transferred to 8 in. diameter pots in a glasshouse and kept there at least eight weeks before being used for experimental purposes. Temperature and humidity in this storage glasshouse were not controlled. After treatment, all seedlings were randomly placed in another glasshouse in which the temperature was maintained at approximately 20°C and the humidity at 60% R.H.

Surface sterilization

Most methods of surface sterilization are obviously unsuitable for use on plant parts which one does not want to damage. An attempt was made, however, to sterilize the surface of pine needles using 70% ethenol. This was reported by Basham (1957) to be suitable for use on woody tissues. The alcohol caused permanent wilting of needles however and as absolute sterilization of the surface was not in any case necessary, a method of partial sterilization was evolved which did not adversly affect the needles. This was achieved by immersing the stem and foliage of the seedlings in 0.1% mercuric chloride for one minute. Following immersion in mercuric chloride the seedlings were held upside down under running tap water for three minutes and allowed to drain for a further five minutes. This prevented the mercuric chloride being washed down on to the soil. The seedlings were then left 24 h. before further treatment. Some residual mercuric chloride would remain on the needles after this treatment. However the results given in Fig. 20 suggest that this had only a small effect on the final numbers of bacteria.

Preparation of "introduced" micro-organisms

Cultures of micro-organisms were obtained from the needle surface of three trees from Bottle Lake plantation by the maceration and dilution plate technique described in Chapter 2. Suspensions were made of bacteria and fungal spores separately by flooding the appropriate plate cultures with 10ml of sterile water and gently rubbing the surface of the plate with the back of a sterile scalpel to dislodge the micro-organisms. The resulting suspensions were then decanted into sterile

containers.

The composition of the population was determined from plates prepared from these suspensions. These suspensions would contain nutrients from the agar which could increase antagonisms on the needle surface. However this initial investigation was intended only to show whether the micro-organisms in the phylloplane of Pinus radiata did in fact behave as an interacting population which would adjust itself to changing conditions and especially "invading organisms" in a recognizable pattern. Any additional nutrients would be present in such low concentrations that they should not interfere with this object.

Application of "introduced" micro-organisms. This was done using a Shandon laboratory spray gun. This generated a very fine spray which gave an even coverage of the seedlings. Each seedling was sprayed with 4ml of suspension, and was then placed in a glasshouse at approximately 20°C and 60% R.H. It is improbable that the water spray would affect the bacterial count by washing off bacteria since all bacteria recorded in this study are those not removed by an initial 3-5 second wash in sterile water.

In any future work on interactions in the phylloplane it would be important to determine:

(i) the number of micro-organisms sprayed on to the needles (i.e. the numbers in the initial suspensions)

(ii) the number of micro-organisms on the needle within an hour of treatment.

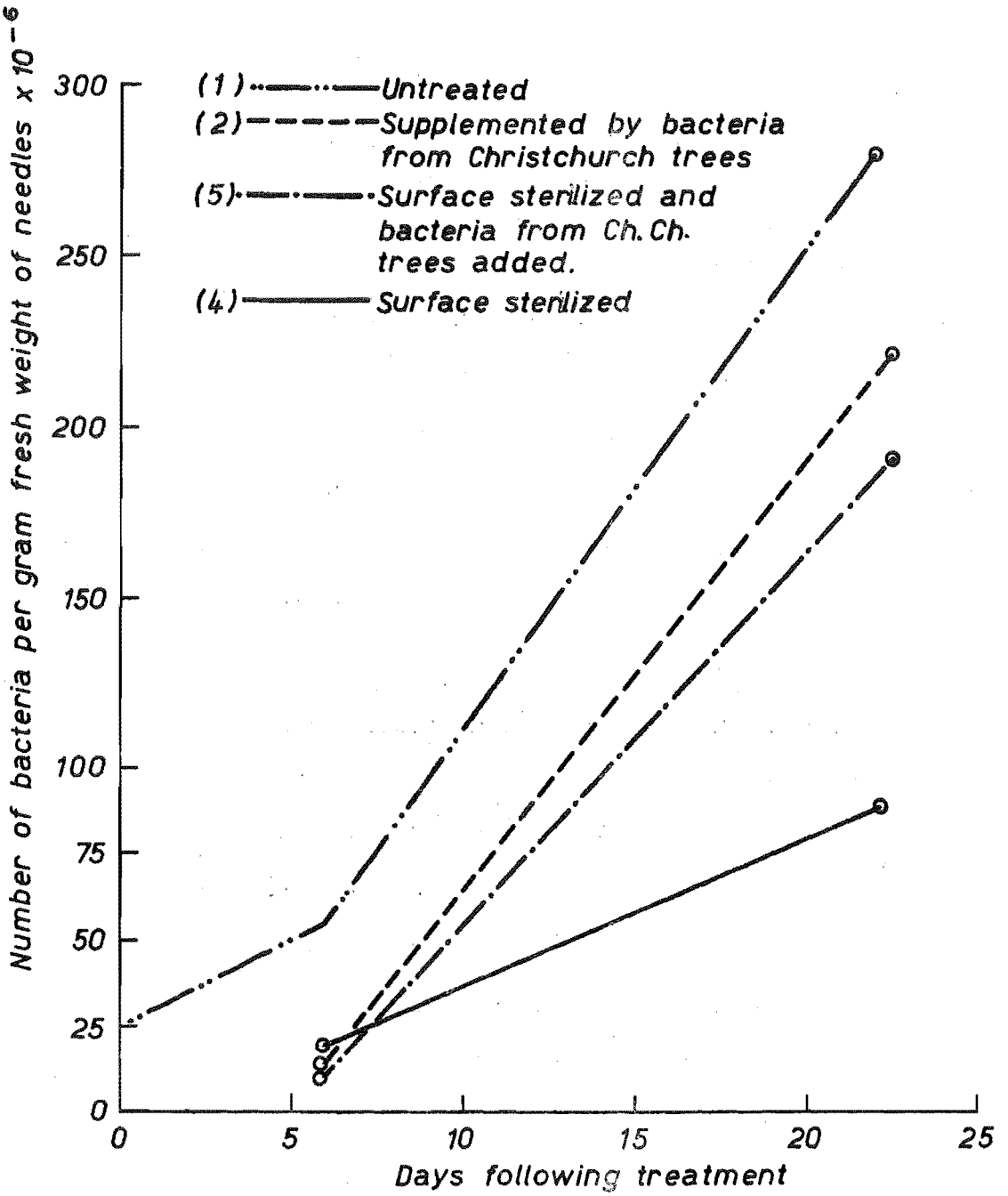


Fig.14 : The effect of artificial treatments on the number of phylloplane bacteria

This information however is not necessary to achieve the limited aims of this trial.

Isolation of resulting populations

After both six days and twenty three days one gram of whole needles was removed from each seedling, macerated and the resulting suspension used to prepare dilution plates for the enumeration and characterization of the micro-organisms.

RESULTS

BACTERIA

"Introduced" bacteria

The composition of the bacterial suspension sprayed on to the seedlings was determined from dilution plates. It was as follows:

coryneforms	60.0%	flavobacteria	6.1%
lactic acid bacteria	11.2%	paracolons	8.6%
pseudomonads	9.0%	cocci	5.1%

This is similar to the results given both in Chapter 3 for other trees from Bottle Lake and in table VII for the untreated controls in this experiment. The proportion of non-pigmented coryneform bacteria found in suspensions from trees grown at Bottle Lake is shown in Fig. 15. This shows about 90% of the coryneform bacteria isolated to be non-pigmented.

Treatment 1 - Untreated seedlings

Total numbers of bacteria. The bacterial counts given in Fig. 14 show the total numbers of bacteria on the untreated needle surface to increase from 25 x 10^6 per gram fresh weight of needles at the time they were placed in the glasshouse to 50 x 10^6 after six days and to 280 x 10^6 after 23 days.

Kinds of bacteria. The proportions of the main groups of bacteria present after 23 days are given in Table VII. These proportions were similar to those recorded at 6 days. The proportion of non-pigmented coryneform bacteria also remained constant at between 27% and 30% of the total coryneform count.

Table VII. Kinds of bacteria isolated from treated needles

Mean % of total bacteria isolated 23 days after treatment.

Treatment	Coryne-form	Lactic acid	Pseudo-monads	Flavo-bacteria	Para-colons	Cocci
1. Untreated	64.0	8.0	8.8	7.2	8.0	4.0
2. Supple-mented	64.8	7.2	8.0	5.4	8.6	6.0
4. Surface sterilized	70.0	6.0	8.4	4.2	8.4	3.0
5. Surface sterilized and supp-lemented	66.0	10.0	8.2	8.0	4.6	3.2

Treatment 2 - Addition of micro-organisms to the normal microflora

Effect on total numbers. The introduction of micro-organisms into a habitat already occupied appeared to cause a nett decrease in total numbers after six days when compared with the controls. During the next seventeen days however the numbers appeared to increase at a similar rate to those on the untreated controls.

Effect on kinds of bacteria. As with the other treatment there was very little alteration in the proportions of the different groups of bacteria present (Table VII). However within the plant coryneform group a marked change can be seen when the treated seedlings are compared with the controls. Six days after spraying with the bacteria from Bottle Lake the proportion of non-pigmented coryneforms is seen to be 83% compared with 30% on the controls. However when the composition was again determined after 23 days the non-pigmented coryneforms accounted for only 27% of the coryneform population(Fig 15).

Treatment 4 - Removal of normal microflora

Effect on total numbers. Fig. 14 shows that compared with the untreated seedlings fewer bacteria were isolated six days after surface sterilization. However after twenty three days the numbers had markedly increased, although to a lower level than the other treatments.

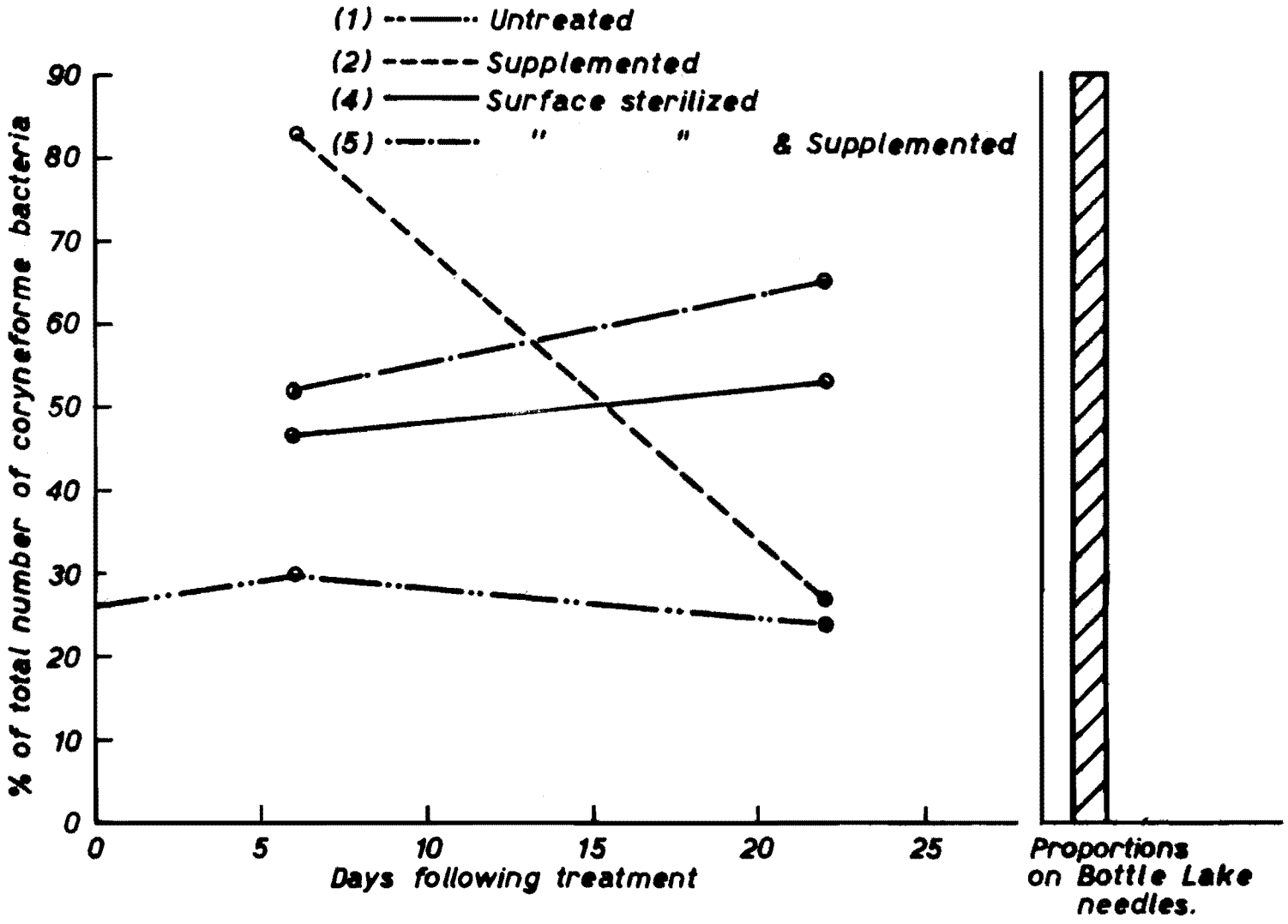


Fig.15: Incidence of non pigmented coryneforme bacteria on needles following treatments

Effect on kinds of bacteria. Table VII shows very little difference in the proportions of the different groups of bacteria present following surface sterilization. Fig. 15 however shows an increase in the proportion of non-pigmented coryneform bacteria although this was not as marked as where bacteria from Bottle Lake were sprayed on to the surface sterilized seedlings.

Treatment 5 - Addition of micro-organisms to surface sterilized seedlings

Effect on total numbers. Fig. 14 shows that following surface sterilization and the addition of bacteria from Bottle Lake there was an initial nett drop in total numbers of bacteria when compared with the controls. After 23 days however the numbers had risen to considerably higher levels than on the unsupplemented surface sterilized seedlings.

Effect on kinds of bacteria. The results given in Table VII again show only very slight changes in the proportions of the main groups of bacteria present following this treatment. Examination of Fig. 15 however shows that within the coryneform group there is an even greater increase in the proportion of non-pigmented forms than was seen in the previous treatment.

MOULDS

Total numbers. As can be seen in Table VIII fungal numbers varied to any extent only in the treatments involving surface sterilization where numbers were initially reduced. After

six days fungal counts showed that where the surface sterilized seedlings were subsequently sprayed with fungi from Christchurch trees, the numbers increased more rapidly than on the un-supplemented seedlings. After 23 days however all treatments and the controls showed similar numbers of fungi on the needle surface.

Table VIII. Numbers of moulds isolated from treated needles $\times 10^{-3}$ per gm

Treatment	Days following treatment 6 days	23 days
1. No treatment	52	45
3. Supplemented	59	53
4. Surface sterilized	15	53
6. Surface sterilized and supplemented	28	49

Kinds of moulds. The kinds of moulds varied little with the different treatments. Although the suspension of moulds used to supplement the normal microflora and sprayed on the surface sterilized seedlings contained large numbers of Penicillium and Cladosporium spores, these fungi did not establish themselves on the needles in numbers greater than appeared on the controls. The fungal population on the control seedlings consisted of Cladosporium 11%, Penicillium 5%, Stemphylium 10%, Pestalotia 4%, Cephalosporium 4%, other sporing fungi 6%, non-sporing fungi 60%. After all treatments these proportions remained essentially the same.

DISCUSSION

Untreated seedlings

External conditions were possibly responsible for the increase in numbers of bacteria on the surface of all needles throughout the experiment since numbers increased at a similar rate on untreated as well as treated needles between 6 and 23 days. The temperature in the glasshouse was maintained at 20°C and the needles were covered with water from the spray which maintained the humidity at intermittant periods. These conditions were shown by Leben and Daft (1967) to allow a rapid increase in bacterial numbers on leaves of a variety of hosts and this factor is discussed further in Chapter 7.

Effect of removing the normal microflora

A study of the microflora on the needles following surface sterilization showed it to be composed of the same kinds of bacteria in similar proportions to those found on the untreated needles. The only indication that the populations may have been different was the difference in the proportion of pigmented to non-pigmented coryneform bacteria. In the untreated seedlings non-pigmented forms comprised 20% of the coryneform isolates whereas after surface sterilization 47% were non-pigmented.

The overall similarity in the composition of the population before and after surface sterilization under different conditions of temperature and humidity suggests that some factor in the host may determine the composition of the population of bacteria resident on its leaves. This would only

influence micro-organisms growing on the needle.

A later examination of this treatment may have been desirable as numbers may not have increased sufficiently over the 23 days for competition and interaction between micro-organisms to be significant.

Addition of micro-organisms to surface sterilized seedlings

The bacteria sprayed on to the seedlings were known to be capable of colonizing the phylloplane. The results of this section of work demonstrate their ability to establish themselves on the seedlings used in this trial. Where bacteria were added to the surface sterilized seedlings the increase in numbers following the initial drop was more rapid than on the un-supplemented seedlings. The proportion of non-pigmented coryneform bacteria in this population was 67%. This is higher than that on the surface sterilized and un-supplemented seedlings (52%). This suggests that in the absence of an established population some of the introduced bacteria multiplied and established themselves in the available habitat.

Addition of micro-organisms to the normal microflora

The nett drop in total numbers following the introduction of large numbers of bacteria into an already occupied habitat is not easily explained.

The disruption of the complex interactions between the micro-organisms may temporarily slow the growth rate of the established population for a period. However this disruption could not occur without the growth of the "invading" micro-organisms. Other studies reported in this thesis suggest that

the un-pigmented coryneform bacteria predominant on trees from Bottle Lake are effective in interactions. Figs. 17 and 18 (pp 94 and 95) show a reduction of both Aureobasidium pullulans and Gram-negative bacteria complementary to the increase in coryneforms on developing needles at Bottle Lake. Moreover where bacteria from Bottle Lake (90% non-pigmented coryneforms) were sprayed on the test seedlings (26% non-pigmented coryneforms) the resultant population five days after spraying was almost identical to that found in the suspension from Bottle Lake trees. Antagonistic interactions such as this might help account for the temporary drop in numbers.

After 23 days a return to a more stable population is suggested by two factors; the increase in bacterial numbers and the readjustment in the proportions of pigmented : non-pigmented coryneform bacteria to those existing on the un-treated needles.

The results reported in this section support the hypothesis that the bacteria isolated from the needle surface are active in this habitat and lead to the conclusion that the bacteria found on the surface of Pinus radiata needles are part of a "phylloplane population".

The bacteria saprophytic on the needle surface may therefore be an important factor in determining whether an organism can grow and multiply in the phylloplane. This is important both from the point of view of the establishment of potential pathogens and where the possibility of biological control by spraying with micro-organisms is indicated. The success of either depends on the ability of the organisms concerned to colonize the needle surface.

MOULDS

The fungi isolated from the needle surface in this study did not appear to vary with any of the treatments apart from a reduction in numbers following surface sterilization of the needles. The irregular occurrence of most genera made it difficult to interpret any changes in composition or to attribute them to any one cause. Total counts suggested there might be some interaction between the fungi and with other micro-organisms on the needle surface, since needles subjected to all four treatments finally supported a similar number of fungi. The introduction of supplementary fungi able to grow in this habitat did not increase the numbers. Some factors must set an upper limit on total numbers and since the organisms introduced were capable of colonizing the needle surface, this limit must include the availability of nutrients, a factor controlled to a large extent by the organisms already utilizing the habitat.

Thus the fungal population may also be influenced by the other organisms in the phylloplane and the mycoflora along with the bacteria on the needle surface may act to regulate the organisms invading and establishing themselves in this habitat.

The results reported in this chapter suggest that the micro-organisms present on the needle surface do interact with each other and so constitute a "phylloplane population". These micro-organisms, particularly the bacteria may therefore play an important role in determining which organisms may colonize this habitat.

CHAPTER SIX

COLONIZATION OF EMERGING NEEDLES

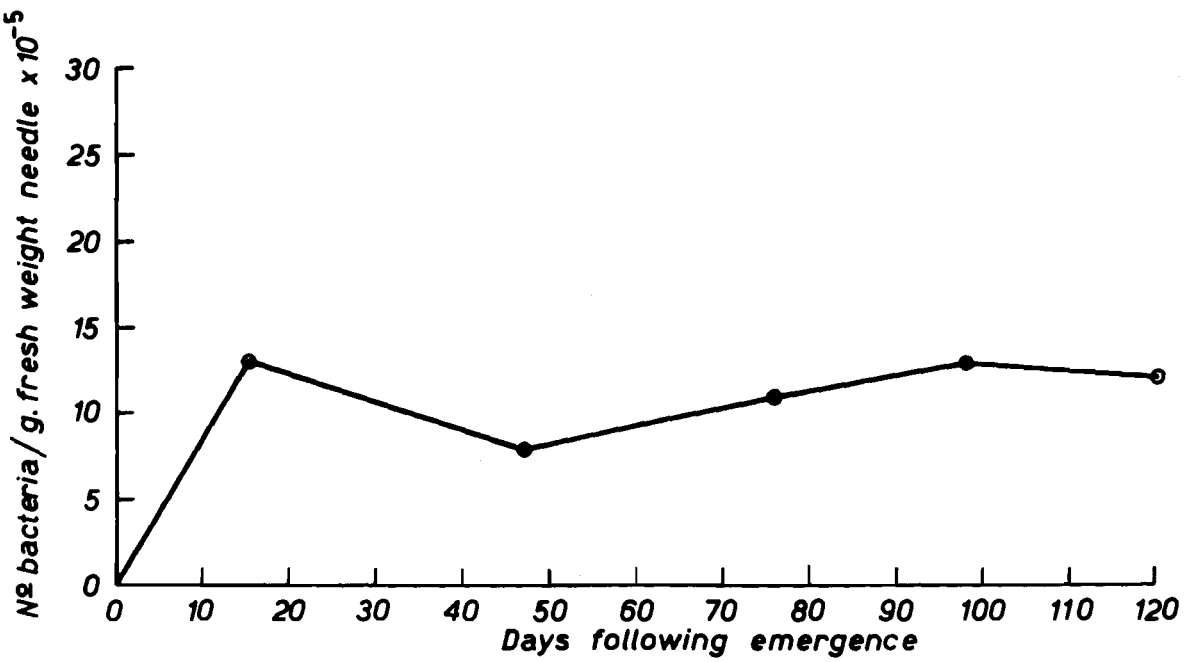
EXPERIMENTAL DESIGN AND METHODS

The population of micro-organisms on mature needles of Pinus radiata was described in Chapter 4. It is probable that a dynamic equilibrium exists between such a population, the host tree and the environment. One aspect of this relationship which has not been investigated in many previous studies of the phylloplane is the colonization of developing leaves. For this reason P. radiata needles were examined periodically as they emerged to see when they were colonized and to note any changes in the microflora which might occur before the characteristic population described in the previous chapters was achieved.

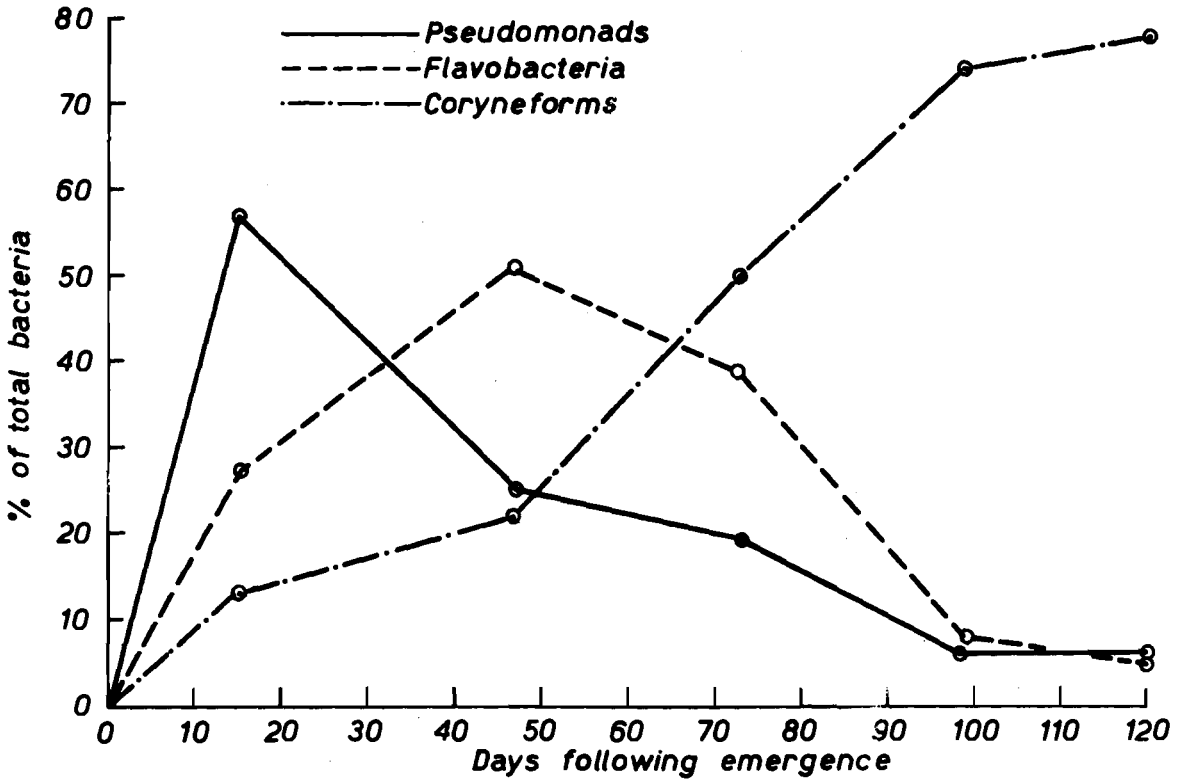
Microflora on unemerged needles

The young needles of P. radiata are formed within an enclosed sheath from which they grow during development. This sheath then remains enclosing the base of the fascicle as it matures.

The first experiment was designed to investigate the micro-organisms on needles prior to emergence from the fascicle sheath. On September 25th thirty sheaths were removed aseptically and prints of the newly exposed needles made on agar plates. Needles were plated on soil extract agar, malt extract agar and Martin's medium to allow the growth of bacteria, yeasts and fungi respectively. The



(a) total numbers



(b) bacterial types

Fig. 16 Bacteria on emerging needles

plates were incubated for two weeks at 25°C and examined daily.

Microflora on emerging needles

Further samples were collected from the same tree on October 4th, November 6th, December 2nd, December 28th and January 18th. On each day the fascicle sheaths were removed and one gram of needles macerated. Separate dilution plate series were then poured for bacteria, yeasts and moulds. Leaf prints were also made to determine the spread of micro-organisms on the surface of the needles.

At the same time 18-month-old needles were collected and treated in the same way for comparison with developing needles.

MICRO-ORGANISMS ON EMERGING NEEDLES

Table IX gives the average lengths of the thirty needles used on each sampling date.

Table IX. Average length of needles emerged from the fascicle sheath (cm)

Sept.25th	Oct. 4th	Nov.6th	Dec.2nd	Dec.28th	Jan.18th
0	1	3.5	5	6.5	7.5

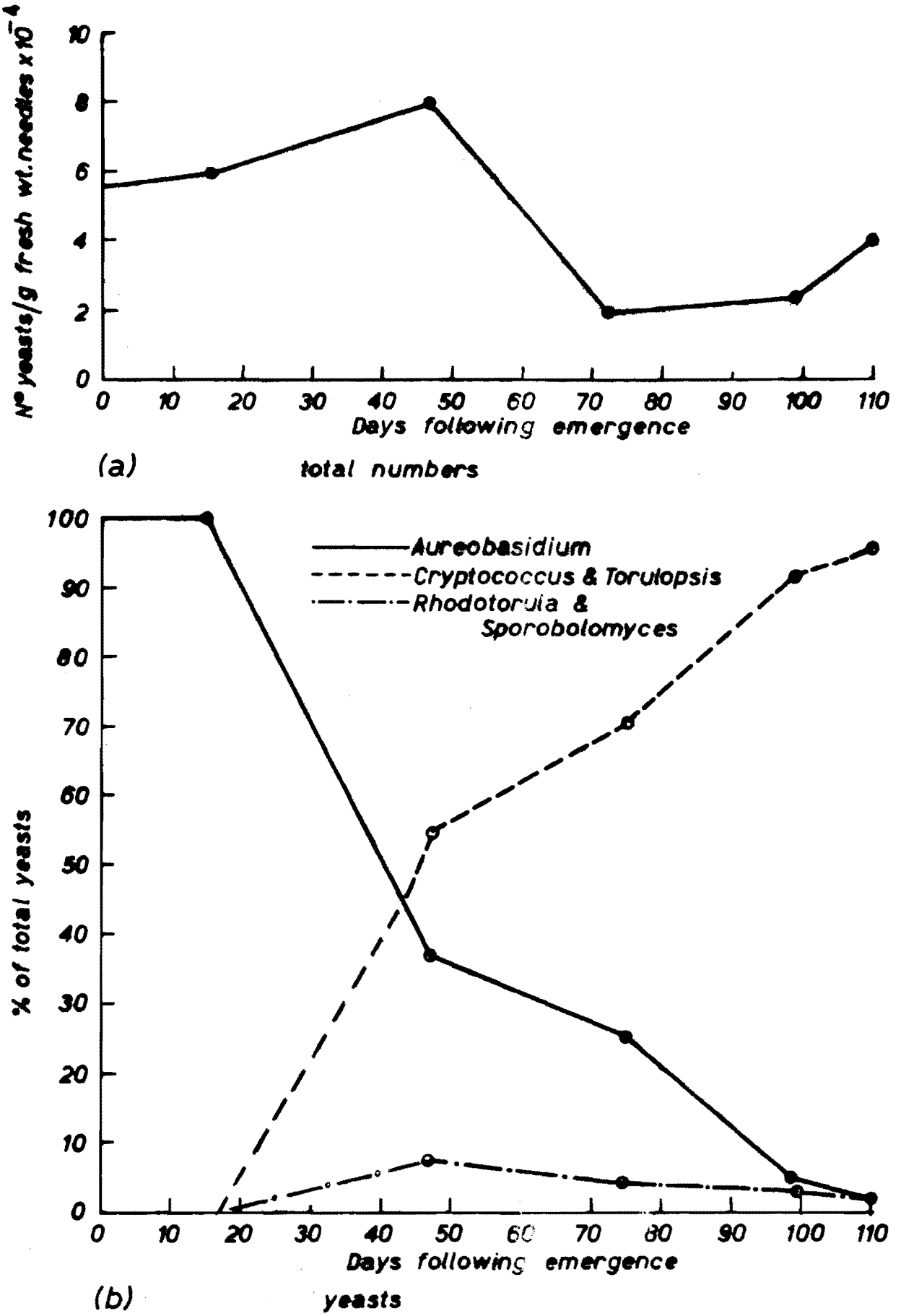
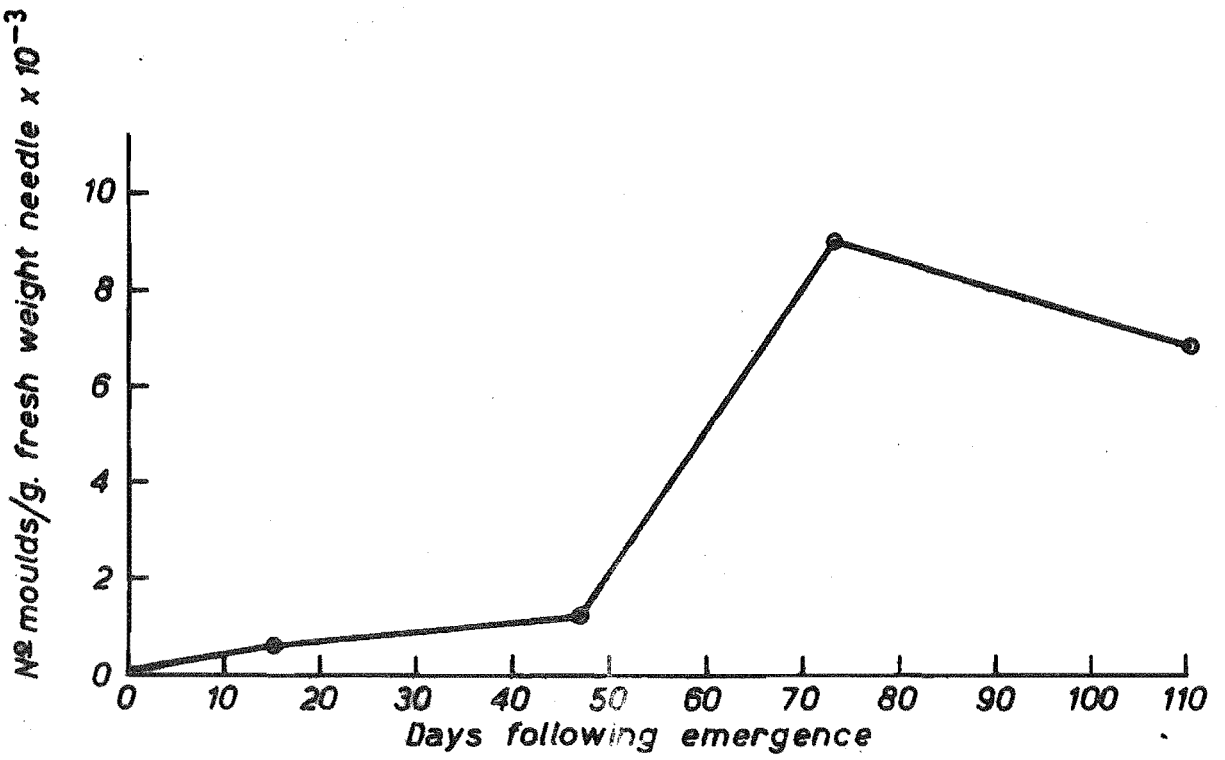
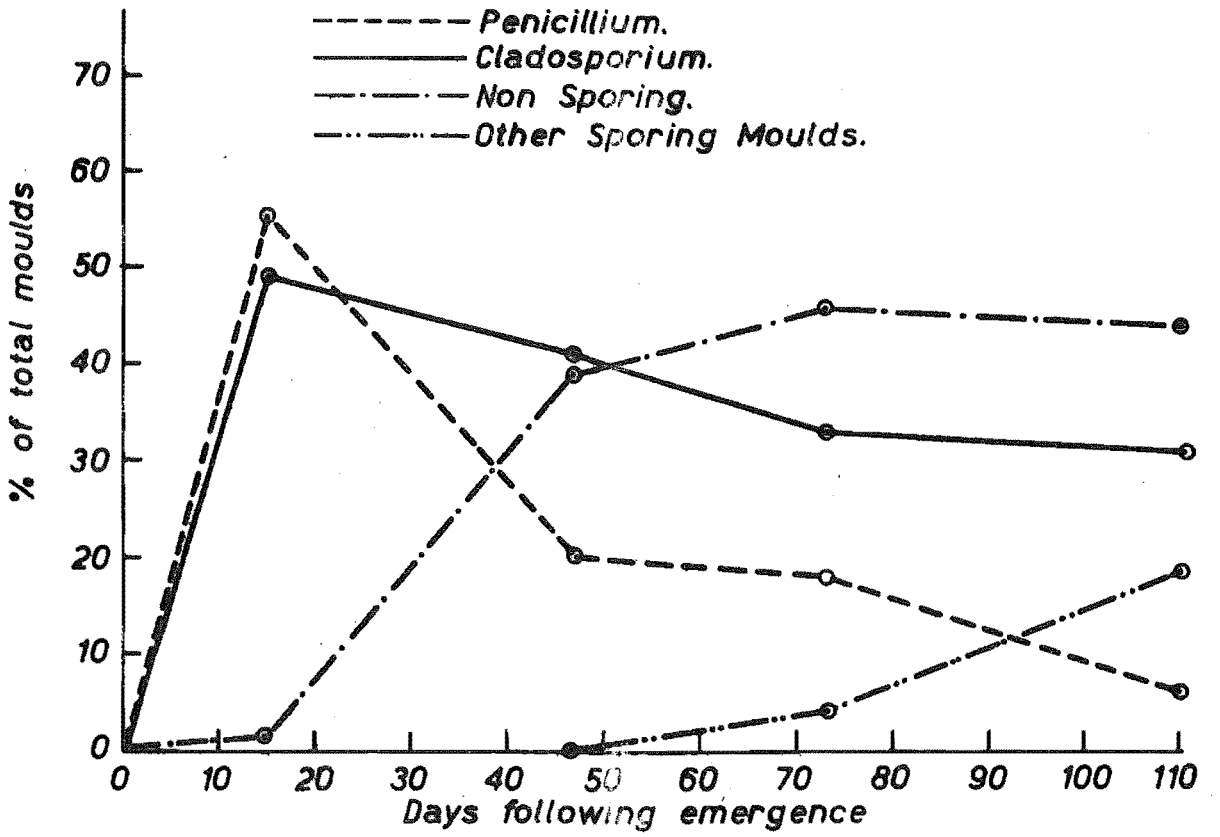


Fig. 17 Yeasts on emerging needles



(a) total numbers.



(b) main groups occurring

Fig.18 Moulds on emerging needles

Dilution plates

Estimated total numbers of bacteria, yeasts and moulds over the period of this survey are shown in Figs. 16, 17, 18 respectively. It is apparent that the needles were free from bacteria and moulds while still in the fascicle sheath. However they became rapidly colonized by a succession of micro-organisms following emergence, until about 100 days after the start of emergence the composition of the microflora was similar to that on the mature needles.

In contrast high numbers of the yeast-like fungus Aureobasidium pullulans were present on the needles within the sheath. Following emergence however there was a steady drop in numbers of this yeast and an increase in the count of Cryptococcus and Torulopsis, genera characteristic of the surface of mature needles. The red-pigmented yeast genera Rhodotorula and Sporobolomyces also became apparent after the needles had broken out of the sheath, but they never became numerous (Fig. 17). Aureobasidium pullulans has been included with the yeasts both because of its yeast-like growth and because it was isolated more readily on glucose-peptone agar, (the medium selective for yeasts) than on Martin's medium which was selective for moulds.

Leaf prints

The leaf prints also indicated that no bacteria or moulds occurred on the needles while they were still within the fascicle sheath. Moreover, that part of the needle within the sheath remained free from bacteria and filamentous fungi for fifty days after emergence of the fascicle. After this time however bacteria and fungi colonized the base of the needle as well as the exposed portion.

The leaf prints also showed that during the early stages of development not all the needles were colonized at the same time.

After 15 days: Bacteria. Fifteen days after emergence four out of 20 needles supported no bacteria, three were colonized by pseudomonads, three by flavobacteria and seven by both bacteria.

Yeasts. The yeast prints showed seven out of 20 needles to support only Aureobasidium pullulans, while the remaining 13 needles showed growth of A. pullulans, Cryptococcus, Torulopsis and Rhodotorula.

Moulds. Eleven out of 23 needles supported Cladosporium alone, six were colonized by Penicillium and three needles remained free from moulds.

After 47 days: Bacteria. All needles were colonized by a mixture of pseudomonads, flavobacteria, and Gram-positive bacteria.

Yeasts. This population was also a mixture of the genera shown in Fig. 17.

Moulds. After 47 days there was a greater variety of moulds on each needle including Cladosporium, Penicillium, Aspergillus, Botrytis, Cephalosporium and Alternaria and some non-sporing moulds.

Microflora on 18-month-old needles

The phylloplane microflora on these older needles varied little over the period of this survey and the composition was similar to that reported in Chapter 3.

DISCUSSION

Colonization of needles by bacteria

One might expect emerging needles to be initially infected by bacteria washed or brushed off other needles, or deposited from the air by gravity or in rainwater. The pseudomonads and flavobacteria which were predominant in the early stages of fascicle emergence do occur in low numbers on the mature needles. Their predominance on the newly emerged needles could be due to their ability to multiply rapidly on a new nutrient source. The Gram-positive coryneform and lactic acid bacteria grow more slowly and could be expected to appear somewhat later. Until the Gram-positive bacteria appeared on the needles the total numbers of bacteria were increasing so apparently not all the available nutrient or space was being utilized. After the first fifteen days the numbers remained fairly uniform (Fig. 16). This suggests there is a readjustment in the population as the proportion of Gram-positive bacteria increases. Since the total numbers of bacteria do not change it appears they establish themselves at the expense of the Gram-negative bacteria.

It is also of interest to note that as the Gram-positive bacteria increase (Fig. 16) there is a corresponding decrease in the numbers of Aureobasidium pullulans on the needles (Fig. 17).

Colonization of needles by yeasts

The occurrence of Aureobasidium pullulans on needles inside the fascicle sheath suggested that the conditions within the sheath and the lack of competition from other micro-organisms allowed rapid growth of the yeast. However once the needles grew from the sheath and were exposed to the atmosphere other types of organisms, including the yeasts more common on mature needles, began to invade the phyllo-plane. The population of A. pullulans declined to very low numbers as Cryptococcus and Torulopsis increased in number. It is impossible to say at this stage whether the drop in A. pullulans was caused by the increase in any one group of organisms (e.g. other yeasts or Gram-positive bacteria) by the increase in total numbers of micro-organisms over this period or by other factors not studied.

Colonization of needles by moulds

As with the bacteria the most likely sources of moulds available to utilize the new habitat provided by the growing needle are the air and other needles. The common occurrence of Penicillium both on the developing needles and in the air suggest that the primary source of fungi may be the air, since this fungus accounts for only a small proportion of the popu-lation found on mature needles. As the needles aged a variety of moulds appeared as the slower growing or less common fungi colonized the needles. The mycelia of these fungi then grew over the needle surface and eventually under the fascicle sheath.

CHAPTER SEVEN

FACTORS INFLUENCING THE PHYLLOPLANE POPULATION

EXPERIMENTAL DESIGN

It has been suggested in the previous chapters that, within the conditions experienced the availability of nutrients on the needle surface may be a major factor determining the size and composition of the phylloplane microflora of P. radiata. The results supporting this hypothesis are:

(i) There only were slight changes in the composition of the phylloplane microflora over a wide range of physical conditions (Table VI, p. 50). This suggests some relationship with the host - possibly nutritional.

(ii) Seasonal changes in numbers of bacteria cannot be directly correlated with changes in temperature, humidity and rainfall (Fig. 10, p.57). Some other factor must cause the fluctuation in numbers.

(iii) Numbers of bacteria were higher on older needles (Fig. 4, p.44) shown to be more susceptible to leaching of nutrients than young leaves (Tukey, 1966).

(iv) The phylloplane microflora varied little on trees grown in different localities (Fig. 6, p.45). This again suggests that the relationship with the host tree may be more important in determining the

composition of the phylloplane microflora.

The work reported in this chapter was designed to investigate more closely the relative importance of various factors in the environment in determining the numbers of micro-organisms on the leaf surface.

The effect of humidity

(a) In the field

First year needles on the lower branches of five healthy eight-year-old trees in Bottle Lake Plantation were covered with plastic (polyethylene) bags and left for 24 h. At the end of this period these branches were removed as well as similar portions from five untreated trees.

Dilutions were prepared from 2g samples of needles from each tree and 10^{-1}, 10^{-3}, and 10^{-4} dilutions plated for enumeration of bacteria, yeasts and moulds.

(b) Under conditions of controlled humidity

More precise information on the effect of humidity on the phylloplane population of *P. radiata* seedlings was sought by placing them in conditions of known humidity.

Ten one-year-old seedlings were put in a McDonald Aquatron inoculation cabinet in which the humidity was maintained at 100% R.H. by an intermittent fine mist. This kept the leaf surfaces wet. Ten others were placed in a glasshouse at Forest Research Institute, Rotorua maintained at 60-70% R.H. by an intermittent fine spray. Humidity and temperature were recorded by means of a thermohygrograph. Another ten seedlings were maintained in a similar glasshouse at 20-30% R.H. In each case the temperature remained between 13°C and 18°C.

After three days 1g of needles from each seedling was macerated and dilution series prepared, as described in Chapter 2.

The effect of temperature

Five one-year-old seedlings were placed in each of three temperature controlled growth rooms constructed in the Botany Department of the University of Canterbury. These were maintained at 1 - 4°C, 10 - 13°C and 25 - 29°C respectively for seven days. At the end of this period 1g of needles was collected from each seedling, macerated, diluted and plated as above. After 14 days the micro-organisms were counted and characterized.

The influence of needle leachate on bacteria

Nutrients produced by the needles appeared to be a limiting factor in the phylloplane. The following experiment demonstrated the effect of the inclusion of needle leachate into the media on which the bacteria were isolated.

Two grams of needles were collected, macerated, diluted and the resulting suspension dispensed into 16 petri dishes. Four dishes were poured with:

(a) nutrient agar (Difco)

(b) nutrient agar plus 10ml/1needle leachate

(c) soil extract agar (Bunt and Rovira, 1955)

(d) soil extract agar plus 10ml/1 needle leachate.

The plates were then incubated at 25°C for 14 days and the colonies counted.

THE EFFECT OF HUMIDITY

(a) Treatment with plastic bags

Fig. 19a shows the number of bacteria isolated from needles from branches covered with plastic bags, and from untreated branches. There was no difference between these treatments, either in numbers or kinds of bacteria.

Fungal numbers (including yeasts) showed a similar lack of response to the conditions of high R.H. found within the plastic bags (see Table X).

Table X. Number of fungi from needles enclosed in plastic bags.

Number per gram fresh weight of needles x 10^{-3}

	Tree a.	Tree b.	Tree c.	Tree d.	Tree e.
Plastic bags	83	71	88	82	71
Control	76	66	81	84	76

The kinds of fungi were similar in each case and similar to those reported in Chapter 3.

(b) Humidity controlled conditions

Fig. 19b shows an increase in the number of bacteria on leaves kept at 100% R.H. as compared with those kept at 20 - 30% R.H. and 60 - 70% R.H.

The proportions of the different groups of bacteria varied only slightly at each humidity tested. The results

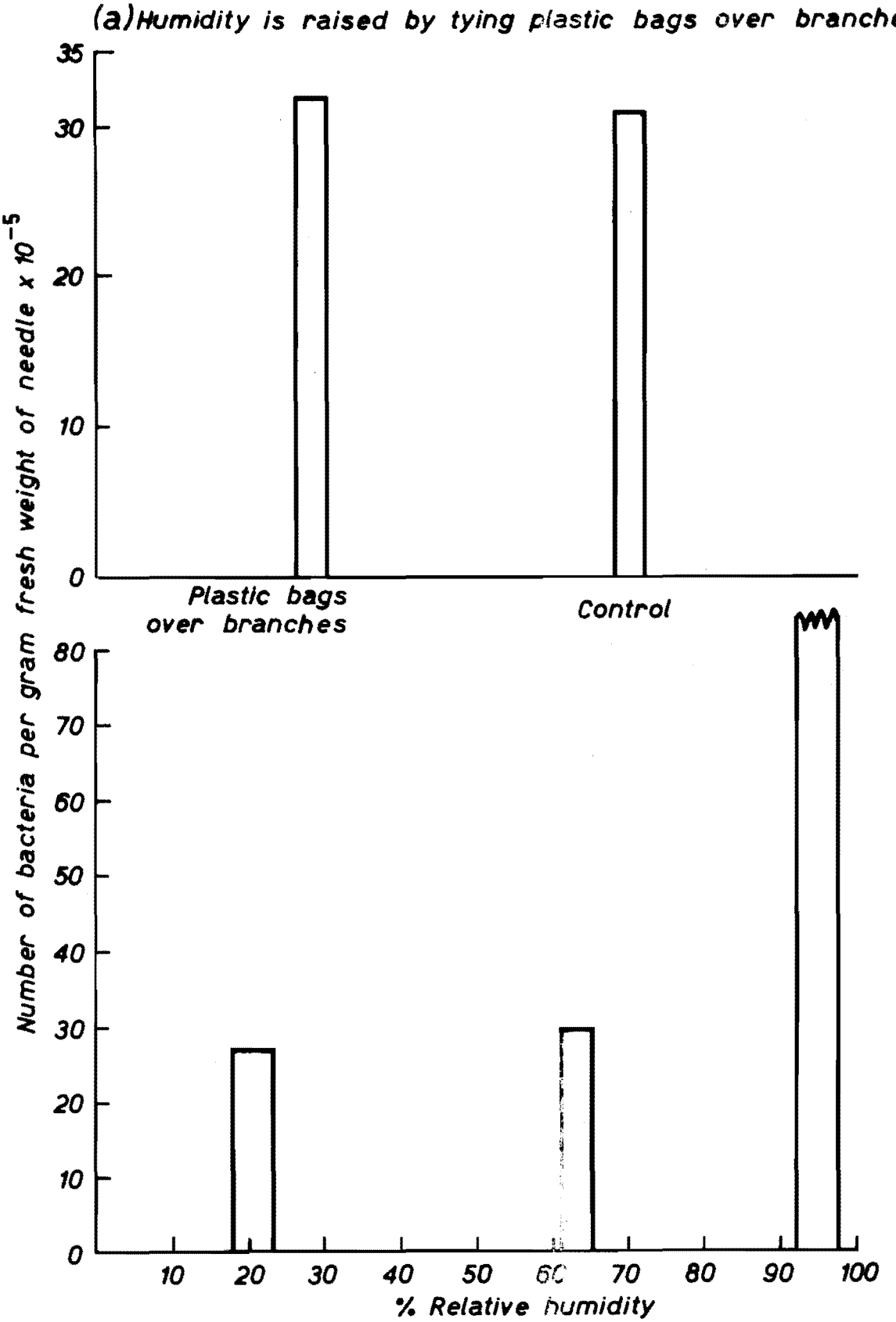


Fig. 19: Effect of Moisture on total numbers of Bacteria

are summarized in Table XI.

Table XI. Kinds of bacteria on needles kept at different humidities.

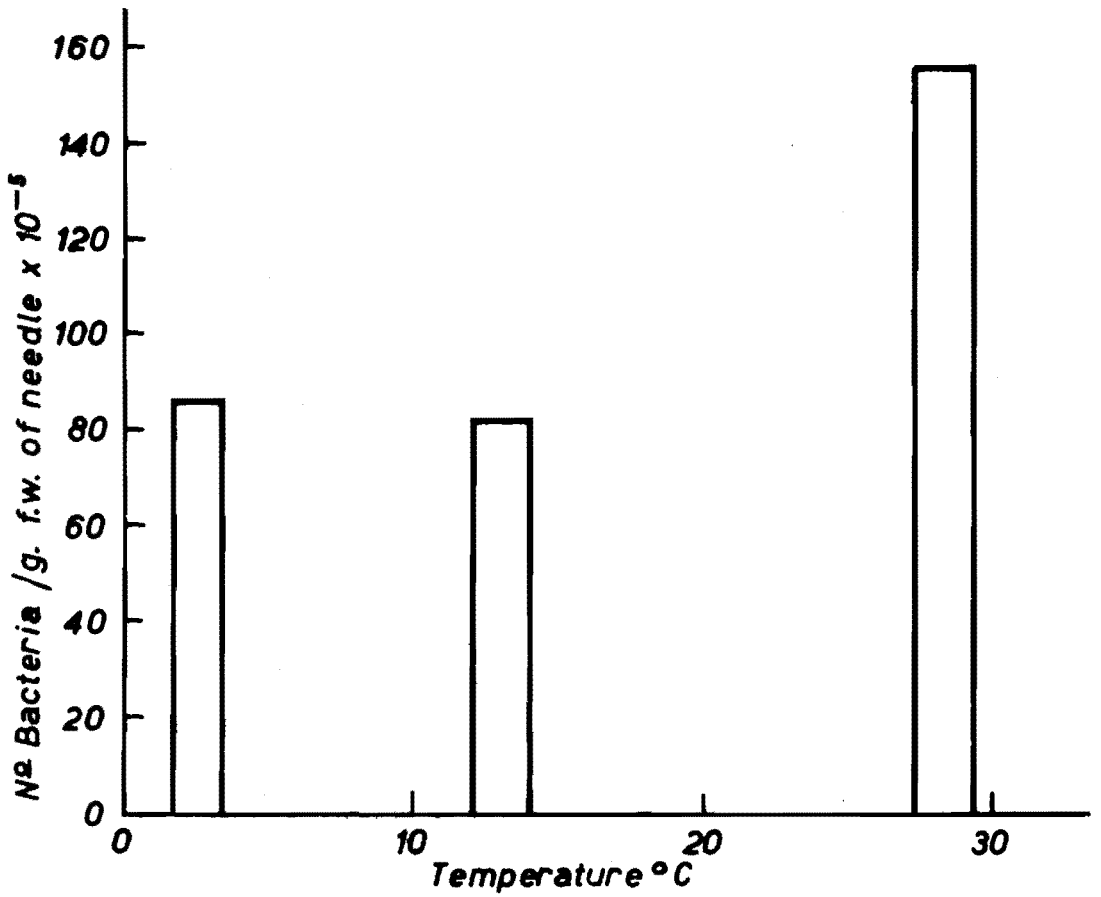
Relative Humidity %	% of Total Number				
	Gram +ve rods	Pseudo-monads	Flavo-bacteria	Paracolons	Cocci
20 - 30	80	8	6	4	2
60 - 70	75	15	6	3	1
100	81	11	5	1	2

The number of fungi isolated from the needle surface at each of the humidities tested showed a greater variation between both numbers and kinds of fungi from the same sample than occurred between different samples. Table XII shows the kinds of fungi isolated at different humidities.

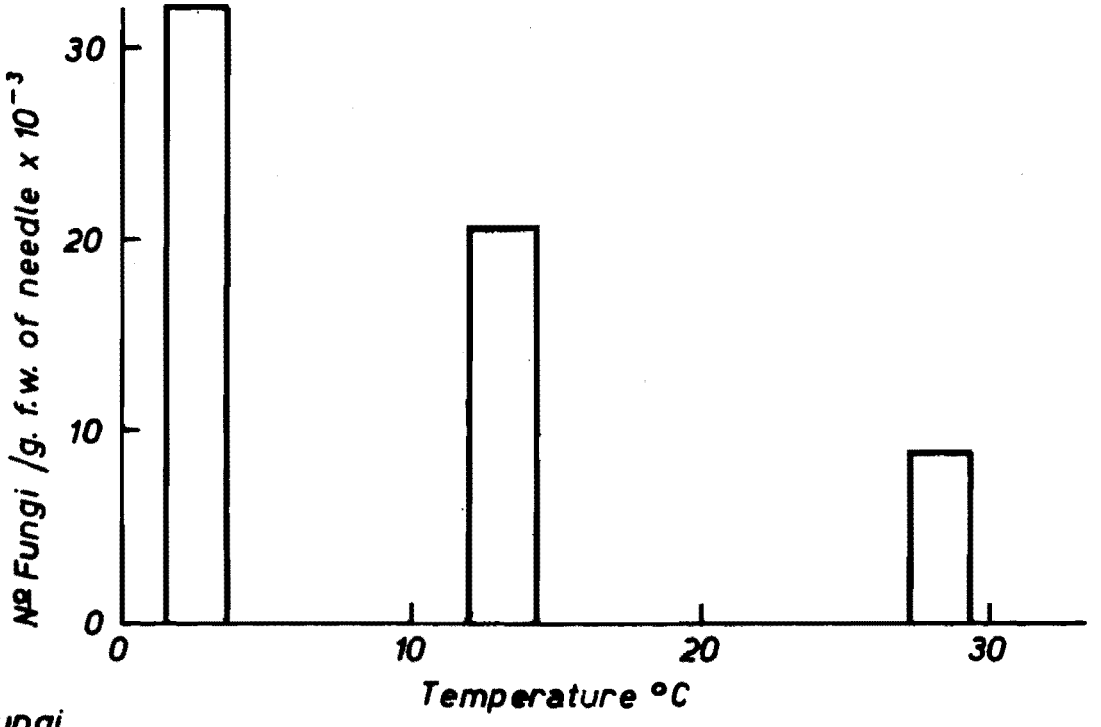
Table XII. Kinds of fungi on needles kept at different humidities.

Average numbers per g fresh weight x 10^{-3} for five seedlings

Relative Humidity %	Cladosporium	Penicillium	Other sporing fungi	Non-sporing fungi
20 - 30	40	9	12	51
60 - 70	63	10	21	37
100	54	6	16	35



(a) Bacteria



(b) Fungi

Fig.20: Effect of Temperature on number of micro-organisms in the phylloplane.

THE EFFECT OF TEMPERATURE

Fig. 20 shows the average numbers of bacteria and fungi isolated from the needles of seedlings grown in a temperature regime of 1 - 4°C, 10 - 13°C and 25 - 29°C. At the two lower temperature ranges bacterial numbers did not vary greatly. There was however an increase in numbers at the higher temperatures. The fungi decreased in numbers from the lower temperatures to the higher temperatures.

Characterization of the isolates demonstrated that neither the kinds of micro-organisms nor the proportions in which they occurred varied over the range of temperatures tested. These proportions are similar to those described in the previous experiment and in Chapter 3.

THE EFFECT OF NEEDLE LEACHATE

The results are summarized in Fig. 21. This shows a marked increase in the number of bacteria growing on the media containing 1% needle leachate. This increase in numbers was brought about by a general increase in all groups of bacteria.

Table XIII shows the effect of needle leachate on the kinds of bacteria isolated.

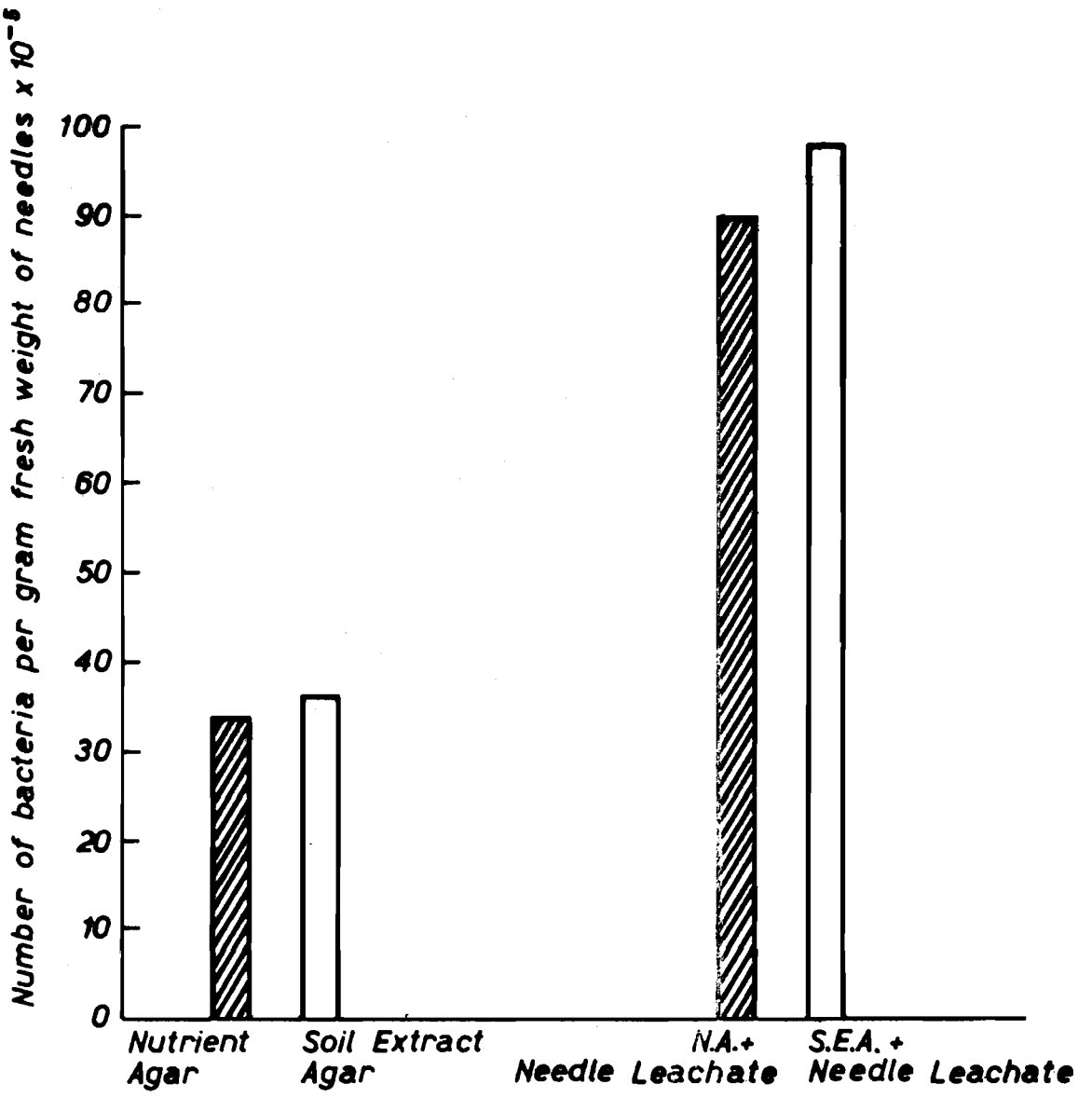


Fig. 21: Effect of Needle Leachate on total numbers of Bacteria.

Table XIII. Effect of needle leachate on kinds of bacteria

% of total bacteria present

Bacteria	Gram +ve rods	Pseudo-monads	Flavo-bacteria	Paracolons	Cocci
Nutrient agar	69	12	9	6	4
Soil extract agar	77	7	6	8	2
Nutrient agar + leachate	78	9	4	4	5
Soil Extract agar + leachate	72	12	7	6	3

DISCUSSION

Humidity

The two experiments yielded apparently conflicting results. The plastic bags raised the R.H. to near 100% without any significant increase in numbers of micro-organisms on the needle surface. However when seedlings were placed in an inoculation cabinet at 100% R.H. which kept the needle surfaces wet, there was a notable increase in bacterial numbers.

Consideration of the methods used to raise the humidity is important in interpreting the results. In the case of the seedlings the humidity was raised using a very fine mist at intervals. Good and Tukey (1966) have shown that an important effect of such misting is to increase the concentration of leached nutrients on the leaf surface. The

presence of high humidity without free water on the needles is said not to have this effect however, and although moisture accumulated on the surface of the bags the needles within appeared to remain dry. Thus the higher numbers of bacteria on the seedling needles are probably a response to increased nutrient availability rather than to increased humidity.

The above results combined with the lack of response of micro-organisms to an increase in R.H. from 22% to 65% suggests that under field conditions atmospheric R.H. is not an important factor in determining the growth of micro-organisms on the surface of needles of P. radiata. Dew formation, mist and rain are however likely to be important as these increase the leaching of nutrients on to the leaf surface.

Temperature

The marked increase in bacterial numbers at 25 - 29°C could be correlated with two factors:

(a) The optimum temperature for growth of all bacteria isolated from pine needles was 20°C or over (Breed et al., 1957). Thus the higher temperatures would increase the rate of growth of bacteria providing nutrients were not limiting.

(b) High temperatures have in some cases been shown to increase the amounts of nutrients leached from the leaf surface (Tukey and Morgan, 1963).

It is therefore possible that higher temperatures increased both the rate of bacterial growth and the amount of nutrients available to them. In the field, temperatures

of the order tested by Tukey and Morgan (ibid) occur only in a few summer months and for brief duration, so high temperatures may be of limited importance.

The other temperatures tested (1 - 4°C and 10 - 13°C) are outside the optimum range for the bacteria isolated from the phylloplane so growth would be slower. However although lower temperatures might slow the rate at which the bacterial population increased, other factors, especially nutrient availability, would limit the final concentration of bacteria in the phylloplane. This could help account for the fact that the numbers of bacteria isolated at each of the lower temperatures were of a similar order.

The inverse relationship shown between temperatures and fungal numbers in the phylloplane was similar to that shown in Fig. 8 where fungi were less common in the summer months, increasing to a maximum in the winter. Most fungi have a lower optimum temperature for growth than the bacteria and the lower winter temperatures combined with the decreased numbers of bacteria might allow increased growth of the fungi on the needle surface.

The influence of needle leachate

The results indicated that a greater number of bacteria from the needle surface grew in the presence of needle leachate than on either of the comprehensive media lacking this.

This suggests that availability of nutrients leached from the host may determine the size of the bacterial population on its leaves, and that any factors affecting this would subsequently be reflected in changes in the phylloplane population. This has previously been suggested in the

discussion on the effect of misting.

The results both of the humidity experiment and of the survey in Chapter 3 suggest that bacteria may be more responsive to increased levels of nutrient than are the fungi. This lack of response by fungi to nutrient availability has been reported in other types of habitats. Burges (1960) notes that when leaf fall adds fresh food reserves to the soil, numbers of bacteria and protozoa increase rapidly while fungal numbers are only occasionally seen to respond. Several suggestions may be put forward to explain this:

(a) The fungal hypha has a relatively long life compared with that of bacteria and this combined with its ability to grow after food may mean it is not quite so vulnerable to changes in nutrient availability and does not need to respond quite so rapidly.

(b) It is possible that a form of fungistasis similar to that found in soil may operate on the leaf surface. It has been noted both in Chapter 3 and in this chapter that where bacterial numbers were very high (Fig. 5) fungal numbers were correspondingly low (Fig. 8) and it is possible that these two trends are related. If this is so the rapid increase in bacteria under conditions of high nutrient and high temperature could depress the growth of fungi. This factor could be as important as the direct effect of high temperatures in reducing fungal growth.

(c) Another contributing factor may be the presence of anti-fungal compounds on the leaf surface. Tokin (1960) has discussed the presence of such agents on the surface of a variety of leaves.

Direct environmental effects do not seem to greatly influence the fungal populations within the range experienced under the conditions tested. Thus the lack of response on the part of the fungi to changes in nutrient level must be related either to characteristics inherent in the fungi or to some external agent depressing either their growth or germination of spores. Which of these is in fact responsible could not be shown without extensive studies on the inter-relationships between the micro-organisms on the needle surface.

CHAPTER EIGHT

INTERACTIONS BETWEEN SAPROPHYTIC MICRO-ORGANISMS ON THE NEEDLE SURFACE AND *DOTHISTROMA PINI*.

EXPERIMENTAL METHODS AND DESIGN

Experiments to investigate interactions between saprophytes on the needle surface and *Dothistroma pini* were designed at three levels.

I. Representative types of epiphytic micro-organisms from the surface of *P. radiata* needles were screened by cultural tests for possible antagonistic properties against the pathogen.

II. Bacteria shown to inhibit *D. pini* in culture were grown with the pathogen on detached needles to determine if inhibition occurred under these conditions and at which stage of the infection cycle it was effective.

III. Simple field trials were set up to test the possibility of using antagonistic micro-organisms from the phylloplane to control the disease in the field.

I. Cultural screening for antagonistic properties against *D. pini*

The following organisms were tested for antagonistic properties against *D. pini*.

Group	Organisms	No. of isolates
Bacteria	flavobacteria	10
	pseudomonads	10
	paracolons	10
	cocci	10
	coryneforms	30
	lactic acid bacteria	10
Yeasts	Cryptococcus	5
	Torulopsis	5
	Rhodotorula	3
	Aureobasidium pullulans	3
Moulds	Cladosporium	3
	Penicillium	4
	Alternaria	1
	Mycelia sterilia	6

The bacteria were grown in nutrient broth for four days, the yeasts in peptone broth for seven days and the moulds on malt agar for seven days, all at 25°C.

Plates of 10% malt agar were then closely streaked with D. pini so that the fungus was spread over the whole plate. Three 5mm discs of filter paper were dipped in each broth culture and transferred to a plate inoculated with D. pini as above.

From each plate on which moulds were growing three 1cm discs were removed with a sterile cork borer and transferred

to a plate inoculated with D. pini.

The plates were then incubated at 18°C for fourteen days. Inhibition of the pathogen was recorded after this period.

II. Effect of bacteria on development of D. pini on detached needles

Detached first year needles of Pinus radiata were placed on moist filter pads in sterile petri dishes and inoculated with both the epiphytic bacteria to be tested, and with D. pini conidia.

Micro-organisms tested

Those bacteria exhibiting antagonistic properties to D. pini in culture were further tested on detached needles. These included:

6 flavobacterium isolates

9 pseudomonad isolates.

Also included were 5 isolates of paracolons not antagonistic to D. pini in the previous test.

Preparation of needles

Healthy needles were removed from a 10-year-old P. radiata tree and five needles placed on moist sterile filter paper in each sterile petri dish.

Inoculation of needles

(i) Inoculation with test micro-organisms. The bacterial cultures were washed from the nutrient agar plates on which they were growing with 10 ml of sterile water. An atomiser was then used to spray each bacterial suspension on to 40 P. radiata needles.

(ii) Inoculation with D. pini. Each plate of five needles was then sprayed with approximately 0.5 ml of a suspension of D. pini spores which had been washed off four plate cultures and diluted with sterile water to a concentration of $1x10^6$ conidia/ml. The needles in their moist chambers were then placed on the bench in sunlight and 10 needles of each treatment examined after two, three, six and seven days to determine the effect of the treatments on spore germination and subsequent growth of D.pini.

Examination of needles

The needles were stained using the periodic-Schiff technique (Preece, 1959) and the development of the spores examined microscopically using both incident and transmitted light. Percentage germination was recorded two and three days after treatment. Germination of 50 spores was recorded from each needle by taking random microscopic fields down the length of the needle. Subsequent growth of the germ tubes was studied after three days. A micrometer eyepiece was used to measure the length of the germ tube of 20 conidia taken at random from each needle. Needles collected after six and seven days were examined to note the spread of hyphae over the needle surface.

III. Field trial on control of needle blight

The bacteria used were the same as those used in the previous experiment. This trial was carried out on one-year-old seedlings raised in the Milton nursery of the New Zealand Forest Service. They had been transferred to Rotorua and maintained in pots under glasshouse conditions at Forest Research Institute for two months.

The micro-organisms were grown on nutrient agar and malt extract agar and removed from the plates as described previously.

Using an atomiser, six seedlings were sprayed with each of the microbial suspensions. Ten seedlings to be used as controls were sprayed with sterile water.

After inoculation, on December 2nd, 1969, the seedlings were taken to Compartment 55 in Kaiangaroa Forest - an area heavily infected with D. pini - and placed in two lines one foot apart in random order under the infected trees. On January 13th, 1970 the seedlings showing needle blight symptoms were counted, and on February 17th the degree of infection of the seedlings was estimated. This was done by staff of the Forest Research Institute who estimated the percentage of foliage exhibiting needle blight symptoms.

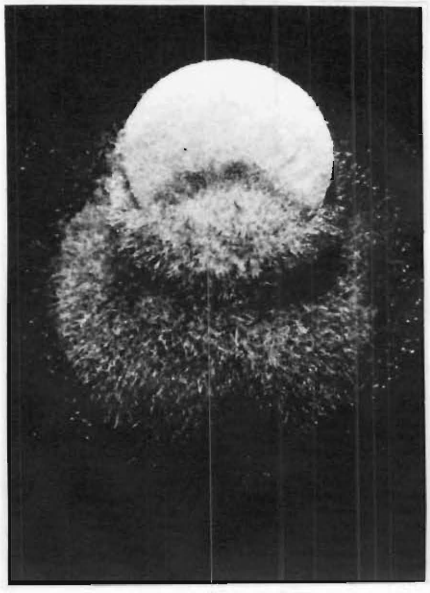
EFFECT OF MICRO-ORGANISMS ON THE GROWTH OF DOTHISTROMA PINI IN CULTURE

Table XIV shows that, of the 80 bacteria tested, 16 were found to inhibit the growth of D. pini in culture. None of the yeasts were effective.

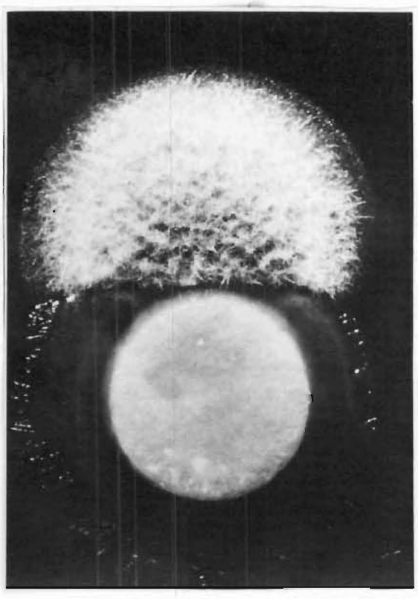
Table XIV. Antagonism of phylloplane micro-organisms against Dothistroma pini

Type of organism	Number of Isolates Inhibition	No Inhibition
Flavobacteria	6	4
Pseudomonads	10	0
Paracolons	0	10
Cocci	0	10
Coryneforms	0	30
Lactic acid bacteria	0	10
Cryptococcus	0	5
Torulopsis	0	5
Rhodotorula	0	3
Aureobasidium pullulans	0	3

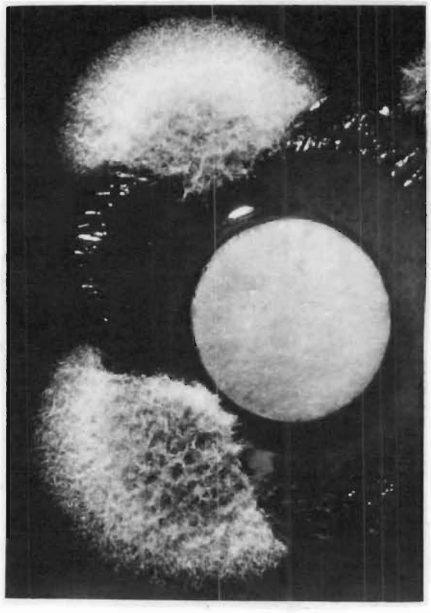
Fig. 22 shows the kind of inhibition that occurred when discs of antagonistic bacteria were placed on top of plates inoculated with Dothistroma pini. No such zone of inhibition occurred round any of the discs of fungi. Both Penicillium and Cladosporium were much faster growing than Dothistroma and tended to overgrow it. They did not appear to prevent growth of the latter however as this could be seen underneath.



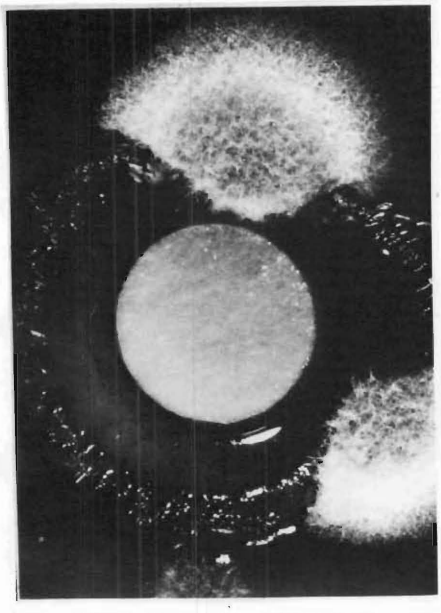
(a) sterile water.



(b) Pseudomonas.



(c) Flavobacterium.



(d) Flavobacterium.

Bacterial Inhibition of D.pini in culture. FIG 22

EFFECT OF BACTERIA ON THE DEVELOPMENT OF D. PINI ON DETACHED NEEDLES

Table XV shows that the addition of high concentrations of bacteria to the needle significantly reduced the germination of D. pini conidia in that habitat. It also shows there was no difference between the effect of the flavobacteria and pseudomonads previously shown to inhibit growth of the fungus in culture, and that of the paracolons which did not affect its growth in culture.

Table XV. Germination of D. pini conidia on detached needles sprayed with bacteria

Treatment	Day 2 %Germination	Day 2 Standard error	Day 3 %Germination	Day 3 Standard error
Flavobacteria	62.1	3.3	69.8	3.6
Pseudomonads	63.4	2.9	70.4	3.5
Paracolons	63.0	3.2	71.4	3.2
Sterile water	75.0	2.5	90.5	2.7

Table XVI shows the length of the germ tubes of the germinating conidia three days after simultaneous inoculation with the bacteria and D. pini. The pseudomonads and paracolons appear to have no influence on growth of the germ tubes when the lengths under these conditions are compared with those on needles sprayed with sterile water. A significant reduction in growth however occurred where the needles were treated with flavobacteria.

Table XVI. Development of germ tubes of D. pini on detached needles sprayed with bacteria.

Treatment	Average germ tube length (μm)	Standard error
Flavobacteria	67.2	2.3
Pseudomonads	78.3	2.4
Paracolons	83.4	3.1
Sterile water	80.6	2.4

Needles collected six and seven days after treatment showed a network of hyphae spreading over parts of the needles. No appressoria had developed and no valid comparison of the development of the pathogen on the differently treated needles could be made at this stage.

EFFECT OF MICRO-ORGANISMS ON THE DEVELOPMENT OF NEEDLE BLIGHT IN THE FIELD

The initial count of infected seedlings after six weeks showed that none of the treatments prevented infection by the pathogen (Table XVII).

Table XVII. Number of infected seedlings six weeks after spraying with bacteria.

Treatment	Number of seedlings	
	Infected	Not infected
Flavobacteria	30	6
Pseudomonads	49	5
Controls	46	8

When the degree of infection of seedlings was estimated after 11 weeks, plants treated with pseudomonads did not differ from the controls, but those treated with flavobacteria appeared to have reduced disease symptoms (Table XVIII).

Table XVIII. Degree of infection of seedlings after treatment with bacteria.

% of seedlings in each category.

Treatment	% Foliage infected			
	<50%	50%	50-70%	>70%
Flavobacteria	0	12	68	20
Pseudomonads	0	5	18	77
Controls	0	0	19	81

DISCUSSION

The cultural tests showed that the growth of some epiphytic bacteria produces a zone in which Dothistroma pini cannot grow. This suggests that the bacteria produce a diffusible substance inhibitory to the fungus. However it has already been stressed that interactions between organisms in the phylloplane are complex. In cannot be concluded that inhibition of an organism in vitro indicates a similar interaction in vivo. The activity of either organism may be modified by the activity of any one or a combination of other micro-organisms in the natural habitat. Antagonism in culture therefore indicates that, given certain conditions, an organism is potentially able to reduce the growth of a second organism. Whether or not this occurs in nature can only be seen by investigating what happens in vivo, i.e. on the needle surface.

The use of detached needles more nearly approximates the natural conditions. Examination of the germination and growth of D. pini conidia on detached needles after spraying with bacteria demonstrated the following:

(i) The presence of these bacteria appeared to reduce conidial germination by 5 - 10%. This occurred irrespective of the ability of the bacteria to inhibit growth of the pathogen in culture.

(ii) The subsequent development of germ tubes was retarded in the presence of those flavobacteria able to inhibit growth in culture. However those pseudomonads shown to prevent growth of mycelium in culture had no effect on growth of germ tubes

on detached needles.

(iii) The resultant growth of mycelium over the needle surface appeared to be extensive in all cases. From visual observation no effect on mycelial growth after seven days could be detected as a result of treatment of the needles with any of the bacteria tested.

From this experiment it appears that the epiphytic flavobacteria tested might reduce germination of Dothistroma spores and the subsequent growth of the germ tubes. However these effects did not appear great enough to reduce subsequent development of mycelium over the needle surface as this was still extensive despite the presence of the bacteria in high numbers.

The final effect of the bacteria on the etiology of the disease however can only be judged in a field trial where the whole disease cycle is represented. For example observation of the amount of growth on the needle surface gives only limited information on its physiological state and its potential for penetration and infection of the needle.

The reduction in the severity of symptoms in the field trial on seedlings treated with the flavobacteria suggested that despite the more complex conditions encountered on the needle surface these bacteria were able to grow and to reduce the ability of D. pini to produce disease symptoms in the host. The cultural tests suggested the production of a diffusible substance inhibitory to mycelial growth. Experiments on detached needles also showed the flavobacteria tested to reduce growth of the germ tubes in the early stages of growth, although no such reductions could be seen at the later stages. A visual assessment such as this however is

only a very rough guide to the actual density of mycelium and gives little information on the physiological state of the mycelium and of its ability to continue the infection cycle.

Experimental evidence given in this chapter does not therefore explain the way in which saprophytic bacteria might influence the growth of potential pathogens and of D. pini in particular. No mechanism is suggested for the reduction of germination of conidia on detached needles in the presence of high concentrations of bacteria. There is no explanation of why both pseudomonads and flavobacteria inhibit growth of D. pini in culture but only flavobacteria are effective on detached needles and in the field. To determine the exact nature of the interaction would require a more detailed study than was possible in this survey. It has however served to emphasize that saprophytic micro-organisms living on the leaf surface are an important part of the environment in which a pathogen must grow and build up its inoculum potential prior to infection.

The phylloplane microflora is therefore a factor worthy of study when control measures are considered. Other studies, e.g. Bier and Rowat (1962), Crosse (1959), Farabee and Lockwood (1958), Riggle and Klos (1970) also suggest that epiphytic micro-organisms might contribute directly to the reduction of disease. However an even more important role might be the part phylloplane micro-organisms play in defence mechanisms of plants resistant to a given pathogen. Removal of these epiphytic micro-organisms by the use of agents such as chemical sprays might, by disrupting the balance of interactions in the phylloplane, remove natural protection against a pathogen previously unable to attack the host.

CHAPTER NINE

SUMMARY AND CONCLUSIONS

The initial work in this study demonstrated the presence of epiphytic bacteria, yeasts and moulds on the surface of healthy Pinus radiata needles. The predominant bacteria belonged to the plant coryneform group. Lactic acid bacteria occurred regularly as well as Gram-negative bacteria such as pseudomonads, flavobacteria and paracolons, including Erwinia herbicola, and some cocci. Most of the yeasts belonged to the family Cryptococcaceae and the predominant moulds were sterile mycelial forms and fungi commonly found in the air spora, particularly Cladosporium and Penicillium. Other sporing fungi were present in low numbers.

The distribution of these micro-organisms under different conditions was studied. This demonstrated that the kinds of micro-organisms present and the proportions in which they occurred were not affected by the range of environmental conditions studied.

A mixture of the above micro-organisms appeared to colonize the whole length of the needles, occurring most regularly in the longitudinal depressions between the epidermal cells. Comparison between the distribution of micro-organisms on different needles showed no pattern of distribution of different types since micro-organisms occurred at random over the length of the needles and differed in locations and concentrations even on needles in a single fascicle.

A study of the development of the phylloplane microflora on newly emerging needles showed the changes in micro-organisms

on the surface of the needles as they emerged from the fascicle sheath. These concluded in the formation of the equilibrium population found on mature needles.

Having studied the composition, distribution and development of the phylloplane microflora, experiments were set up to determine some of the factors influencing this population. These showed:

i. The R.H. of the surrounding air did not greatly influence the population on the needle surface.

ii. High temperatures increased the rate of bacterial growth but reduced the number of fungi isolated.

iii. Needle leachate increased bacterial numbers but did not markedly affect fungal numbers.

These results as well as those on the distribution of micro-organisms under different conditions suggested that the greatest single factor influencing the size and composition of the phylloplane microflora was the amount of nutrients available on the needle surface. Physical factors in the environment were secondary within the range of conditions usually experienced in the field.

The existence of inter-relationships between the component micro-organisms of the phylloplane population was investigated by disrupting the existing balance on the needle surface. This was done both by removing some of the existing population by partial surface sterilization, and by adding micro-organisms from trees grown in a different locality. The resulting changes in numbers and kinds of micro-organisms were studied. These suggested that the micro-organisms on the needle surface, particularly the bacteria, played an important role in determining the composition of the phylloplane population.

The importance of these epiphytic bacteria in the development of D. pini, on the needle surface was examined using both cultural and field tests. The results suggested that micro-organisms were important as an integral part of the environment in which the pathogen grew and developed. The presence of saprophytic bacteria reduced both germination of conidia and subsequent growth of the germ tubes although the final distribution of mycelium on the needle surface was not noticeably influenced. Despite this, some saprophytic flavobacteria when sprayed on to seedlings reduced the severity of needle blight symptoms.

This study constituted a survey of the existence, composition and activity of micro-organisms in the phylloplane and their interaction with the fungal pathogen D. pini. In a study such as this many questions remain unanswered and new questions are posed by the results of the work. The micro-organisms isolated were only partly characterized and more work could be done to further classify these. Methods of isolation were mainly limited to the dilution plate technique, and the use of other methods and media could reveal completely different kinds of bacteria and fungi.

Future work might well include the development of techniques to more clearly determine the distribution of micro-organisms on the needle surface. The scanning electron microscope is the best instrument available for this purpose but in the preliminary work described in this thesis it proved difficult to differentiate bacterial cells from other material on the needle surface.

The changing micro-flora on developing needles has been described but no attempt made to determine the factors

responsible for these changes. Similarly, in the studies of interactions between micro-organisms, changes in populations were described but how these were brought about was not determined. However, the study of factors influencing the numbers and kinds of micro-organisms on the needle surface suggested that nutrient availability was the most important single factor. It is therefore likely that knowledge of the composition of the nutrients and of the factors affecting their accumulation on P. radiata needles would lead to a greater understanding of the development of the phylloplane microflora and of interactions between its component species.

It becomes obvious that investigation of the phylloplane as an environmental niche is still incomplete. The projects described in this thesis achieved their aims insofar as they constitute a comprehensive survey of the composition, development and distribution of micro-organisms on P. radiata needles. Their activity and importance however have only been studied in the broadest sense. A greater understanding of the micro-organisms in this habitat could now result from a move from descriptive studies such as this to more detailed investigations in any of the areas suggested.

These results however also have implications in the field of plant pathology where a potential leaf pathogen must build up its inoculum potential on the needle surface prior to penetration. In some cases the microbial population on the leaf surface might constitute an effective defense mechanism for the host plant. Further investigation of this possibility is necessary for a complete understanding of the host-pathogen relationship. The use of chemical sprays

while conferring protection against a particular pathogen may, by inhibiting the epiphytic micro-organisms, make the host susceptible to other pathogens normally controlled by these. Hislop and Cox (1969) investigated the effects of captan on the saprophytic microflora of apple leaves and showed that it had a marked effect in reducing the fungal flora on buds and leaves. They showed however that when spraying was stopped the microflora returned to near normal in a few months. This factor warrants further study.

On the other hand, as noted by Wood and Tveit (1955) knowledge of interactions between micro-organisms in the phylloplane may suggest means of biological control by introducing antagonistic micro-organisms, by increasing the concentration of epiphytes known to inhibit the pathogen, or by altering the environment so the growth of antagonistic saprophytes is encouraged.

This survey has indicated that it may be difficult to establish saprophytes on needles already colonized, or to alter the proportions in which these occur. However it has also suggested that some degree of protection against D.pini resulted from spraying seedlings with antagonistic flavo-bacteria. A greater understanding of factors controlling the phylloplane population and of the interactions between the micro-organisms is therefore necessary to indicate more clearly the ways in which the phylloplane microflora could by important in the etiology of any particular disease.

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APPENDICES

Appendix I Table XIX Plate counts and Chi square variation for plate counts of bacteria from different sources.

Source of bacteria																	
	Position on tree				Age of tree		Age of needle			Locality of tree			Month of Year				
Replicate	Top	Bottom	Sunny	Shaded	8 yrs	35 yrs	1st yr	2nd yr	3rd yr	Reefton	Rotorua	ChCh.	July	Oct	Nov	Feb	May
Sample 1. 1	37	196	93	266	65	67	77	125	182	52	71	81	143	226	266	65	81
2	43	188	79	258	68	85	71	122	164	56	77	64	125	200	258	68	64
3	45	172	92	260	56	82	67	116	187	47	84	83	128	234	260	56	83
4	32	178	74	270	55	79	83	108	192	43	78	79	138	208	272	55	79
5	38	178	94	244	57	585	106	121	199	53	71	87	122	239	244	57	87
Sample 2. 6	32	205	92	260	51	55	104	128	192	69	86	85	110	218	260	51	85
7	30	203	94	225	56	59	96	125	199	71	85	71	116	217	225	56	71
8	35	201	91	240	52	68	81	107	176	45	72	64	128	212	240	52	64
9	37	193	72	261	46	74	98	143	187	58	86	73	116	203	261	46	73
10	27	186	101	225	62	51	98	130	185	57	91	75	116	220	225	62	75
Sample 3. 11	30	194	94	229	72	75	83	136	187	47	80	71	121	199	229	72	71
12	47	198	97	258	65	67	86	139	197	60	76	67	123	204	258	65	67
13	34	206	85	270	71	63	96	140	204	52	94	64	136	217	270	71	64
14	35	185	83	231	66	70	85	141	184	65	69	75	136	235	231	66	75
15	22	199	79	222	67	54	84	137	162	53	74	77	123	219	222	67	77
mean	34.9	190.8	88.0	248.0	60.6	68.9	86.3	127.8	186.4	55.2	79.6	74.4	125.4	216.0	248.0	60.6	74.4
χ^2	17.9	8.41	12.18	18.16	14.3	19.48	18.1	14.8	10.9	17.9	10.54	11.04	9.98	8.94	18.06	14.3	11.04
p	<10%	<75%	<50%	<10%	<25%	<10%	<10%	<25%	<50%	<25%	<50%	<50%	<75%	<75%	<10%	<25%	50%

If p falls between 95% and 5% the Plate counts fit a Poisson Series.

APPENDIX II

Table XX. Results of Tests Carried out on Gram-positive Bacteria

CHARACTER	Group	% Positive Results Coryneform	Lactic Acid	Cocci
	No isolates	1547	174	89
rods		100	75.3	0
cocci		0	24.7	100
snapping division		73	0	N.A.
pallisading		39	0	N.A.
motile		32±	0	0
acid fast		0	0	0
Gram reaction		36.2(74.8±)	100	60(40±)
10% NaCl tolerance		5	0	17.4
6% NaCl tolerance		42	22.3	47.6
oxidative		0	0	32.3
Utilization of glucose fermentative		72.8S	84.3S	67.7
acid from sucrose		52.2S	73.2S	64.7
acid from lactose		49.3S	94.3	61.4
hydrolysis of gelatin		36.0	0	51.3
reduction of nitrate		53.2	-	58.6
effect on milk		27.3A 26.1Ak 21.4P	63.4P 12.1A	25.0P 53.1A
catalase		100	0	100
oxidase		39.6	-	100
pigmentation		25Y,12R,63N	15Y 85N	10R,42.3N 47.7Y

± = variable reaction
N.A. = Not applicable
S = Slow reaction
A = Acid production
Ak = Alkali production
P = Peptonization
Y = Yellow
R = Pink
N = Non-pigmented

Table XXI. Results of Tests Carried out on Gram-negative Bacteria

CHARACTER	Group	% Positive Results			
		Pseudo-monads	Flavo-bacteria	Para-colons	"Erwinia"
	No. isolates	44	30	28	34
rods		100	100	100	100
motile		100	67.2	69.3	100
polar		100	3.2	-	0
flagella					
peritrichous		0	64.0	-	100
Gram-reaction		0	0	0	0
oxidative		98.0	97.9	0	0
Utilization of glucose					
fermentive		2.0	23.1	100	100
acid from sucrose		74.6	47.2	62.3	69.4
acid from lactose		61.2	31.4	21.3S	63.4
hydrolysis of gelatin		92.3	57.4	61.4	67.3
reduction of nitrate		63.1	49.7	59.1	77.0
effect on milk		-	42A 21.3Ak	37.2A 26.8P	35.1A 24.2P
oxidase		100	100	0	0
pigmentation		85.2N 24.8R	98.4Y 1.6N	100N	100Y
production of water soluble pigment		62.4	0	0	0

S = slow reaction

A = acid production

Ak = Alkali production

P = peptonization

N = non pigmented

R = pink

Y = yellow

APPENDIX III

Table XXII. Numbers of Gram-negative Bacteria isolated under Differing Conditions

		Number isolated from samples of 50			
Source of Bacteria	Group	pseudo-monads	para-colons	"Erwinia"	flavo bacteria
top of tree		2	1	1	2
bottom of tree		2	2	2	2
shaded aspect		3	3	4	2
sunny aspect		2	1	2	-
current year's needles		-	3	-	2
2nd year needles		2	2	2	2
3rd year needles		4	3	1	4
trees 30 yrs		1	2	3	3
trees 10 yrs		4	2	2	2
Bottle Lake trees		4	2	2	3
Reefton trees		5	0	4	4
Kaiangaroa trees		6	4	0	2
June		2	2	0	2
July		3	3	1	2
November		3	3	4	2
February		4	2	2	2
May		4	1	2	3

APPENDIX IV

Table XXIII. Pigmented and Non-pigmented Bacteria from Sunny and Shaded Aspects

Source of Bacteria	% of Total Isolates	
	Pigmented	Non-pigmented
Shaded Aspect	36.1	64.9
Sunny Aspect	40.0	60.0